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RESPIRATORY MONONUCLEAR PHAGOCYTES DURING STEADY STATE AND SARCOIDOSIS

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Respiratory mononuclear phagocytes during steady state and sarcoidosis

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“No one can construct for you the bridge upon which

precisely you must cross the stream of life,

no one but you yourself alone.”

Friedrich Nietzsche

ABSTRACT

Each day, our lungs inhale thousands of liters of air containing not only essential oxygen but also smoke particles, dust, allergens, and potentially dangerous pathogens. In order to tolerate harmless antigens and initiate an immune response against pathogens, a well-organized network of immune cells is required in the respiratory tract. Mononuclear phagocytes (MNPs), comprised of macrophages, monocytes, and dendritic cells, line the respiratory mucosa and are equipped with tools to recognize and rapidly respond to foreign materials. Despite MNPs act as sentinels at the mucosal barrier, infections and inflammation can affect the lungs. Pulmonary sarcoidosis is a T cell-driven inflammatory disease characterized by granuloma formation. The causative antigen is yet to be identified but MNPs are known to be involved in the pathogenesis of sarcoidosis. A theory is that the antigen is taken up by macrophages. Macrophages also produce vast amounts of the pro-inflammatory cytokine tumor necrosis factor (TNF). Dendritic cells on the other hand, take up the antigen and transport it to the lymph nodes where they activate naïve T cells. Circulating monocytes have an important role in cytokine production. However, the role of respiratory monocytes during sarcoidosis is less well studied. We hypothesized that pulmonary MNPs are crucial in maintaining a steady state of anti- and pro-inflammatory processes in healthy individuals. This dynamic process is disturbed during sarcoidosis, thereby contributing to pathogenesis. Hence, our aim was to study MNPs in the respiratory tract during steady state and sarcoidosis.

First, we investigated MNPs in the human respiratory tract. We found profound differences in distribution of seven MNP subsets between blood and the respiratory tract both during steady state and sarcoidosis. We observed an increase in frequencies of blood and respiratory monocytes in sarcoidosis patients compared to healthy controls. Additionally, monocytes from sarcoidosis patients showed an inflammatory profile with upregulation of genes related to inflammatory pathways. Intriguingly, MNPs from the lungs of sarcoidosis patients produced TNF without external stimulation to a significantly higher degree than that of MNPs from healthy controls. In contrast to previous observations, we found that pulmonary monocytes contributed more to TNF production than macrophages. Additionally, we associated higher frequencies of monocytes in the circulation as well as high numbers of intrinsically TNF producing monocytes at time of diagnosis with progressive disease development in sarcoidosis.

In conclusion, we have mapped the MNP network in several anatomical locations of the respiratory tract during steady state and sarcoidosis. We also identified pulmonary monocytes to play an important role in disease pathogenesis in sarcoidosis. That knowledge can help to design new treatment options in sarcoidosis to favor disease resolution and improve quality of life.

LIST OF SCIENTIFIC PAPERS

I. Faezzah Baharom, Saskia Thomas, Gregory Rankin, **Rico Lepzien**, Jamshid Pourazar, Annelie F. Behndig, Clas Ahlm, Anders Blomberg, and Anna Smed-Sörensen.

Dendritic cells and monocytes with distinct inflammatory responses reside in lung mucosa of healthy humans.

Journal of Immunology, 2016, 196(11): 4498-509

II. **Rico Lepzien**, Gregory Rankin, Jamshid Pourazar, Ala Muala, Anders Eklund, Johan Grunewald, Anders Blomberg, and Anna Smed-Sörensen.

Mapping mononuclear phagocyte in blood, lungs and lymph nodes of sarcoidosis patients.

Journal of Leukocyte Biology, 2019; 105:797-807

III. **Rico Lepzien**, Sang Liu, Paulo Czarnewski, Mu Nie, Björn Österberg, Faezzah Baharom, Jamshid Pourazar, Gregory Rankin, Anders Eklund, Matteo Bottai, Susanna Kullberg, Anders Blomberg, Johan Grunewald, and Anna Smed-Sörensen.

Monocytes in sarcoidosis are potent TNF producers and predict disease outcome.

Manuscript

IV. **Rico Lepzien**, Mu Nie, Sang Liu, Avinash Ravindran, Susanna Kullberg, Anders Eklund, Johan Grunewald, and Anna Smed-Sörensen.

Blood and BAL dendritic cells from sarcoidosis patients induce IFNγ producing Th1 cells.

Manuscript

PUBLICATIONS NOT INCLUDED IN THE THESIS

I. Ylva Kaiser, **Rico Lepzien**, Susanna Kullberg, Anders Eklund, Anna Smed-Sörensen, and Johan Grunewald.

Expanded lung T-bet^{+}RORγt^{+} CD4^{+} T-cells in sarcoidosis patients with a favourable disease phenotype.

European Respiratory Journal, 2016 Aug;48(2):484-94

II. Faezzah Baharom, Oliver S. Thomas, **Rico Lepzien**, Ira Mellman, Cécile Chalouni, and Anna Smed-Sörensen.

Visualization of early influenza a virus trafficking in human dendritic cells using STED microscopy.

PLoS One, 2017 Jun 7;12(6):e0177920

III. Mariana Hugo Silva, **Rico Lepzien**, Sebastian Ols, Benita Dahlberg, Johan Grunewald, Karin Loré, Anna Smed-Sörensen, Margarida Correia-Neves, Nuno Empadinhas, Anna Färnert, Gunilla Källenius, and Christopher Sundling.

Stabilization of blood for long-term storage can affect antibody-based recognition of cell surface markers.

Journal of Immunological Methods, 2020 May 6;112792

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LIST OF ABBREVIATIONS

AM	Alveolar macrophage
APC	Antigen-presenting cell
BAL	Bronchoalveolar lavage
BFA	Brefeldin A
BW	Bronchial wash
CCR	C-C chemokine receptor
CD	Cluster of differentiation
cDC	Conventional dendritic cell
CFSE	Carboxyfluorescein succinimidyl ester
CM	Classical monocyte
COPD	Chronic obstructive pulmonary disease
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EBB	Endobronchial biopsy
EBUS-TBNA	Endobronchial ultrasound-guided transbronchial needle aspiration
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
HC	Healthy control
HLA	Human leukocyte antigen
IFNγ	Interferon gamma
IL	Interleukin
IM	Intermediate monocyte
IRF	Interferon regulatory factor
LLN	Lung-draining lymph node

LPS	Lipopolysaccharide
LS	Löfgren’s syndrome
mo-DC	Monocyte-derived dendritic cell
mo-Mac	Monocyte-derived macrophage
MHC	Major histocompatibility complex
μM	Micromolar
MNP	Mononuclear phagocyte
NCM	Non-classical monocyte
Non-LS	Non-Löfgren’s syndrome
PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid dendritic cell
PRR	Pattern recognition receptor
RES	Reticuloendothelial system
RNA	Ribonucleic acid
RNAseq	Ribonucleic acid sequencing
RORγt	Retinoic acid receptor-related orphan receptor gamma t
TB	Tuberculosis
Tbet	T-box transcription factor
TGF-β	Transforming growth factor beta
Th	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor

1 INTRODUCTION

For the human species to exist in nature, evolution has created physical, chemical, and cellular barriers in the body to protect itself from the environment. The field of immunology studies the body's immune response against pathogens. Additionally, the human immune system in its diversity and specificity has developed mechanisms to tolerate the body's own structures in order to protect it from "self-destruction." The immune system is highly complex and the defense or tolerogenic mechanisms do not always work perfectly. Thus, studies of the immune system during healthy and disease conditions are required. Understanding the immune system allows to target and to manipulate it, thereby increasing future therapeutic options.

The immune system spans throughout each organ of the body. It can be divided into an innate component as well as a highly specific adaptive immune system. The innate immune system is evolutionarily older and can be found in all animals. In contrast, the adaptive immune system is a highly specialized adaptation to the environment and is most developed in jawed vertebrates.[1] The immune system is able to recognize and respond to threats, while not damaging self-structures, and to remember the threat. The response to a pathogen can be very powerful and can lead to harmful side effects. Hence, a main task of the immune system is to keep the body in a steady state by balancing the response to exogenous antigens while maintaining organ function.

The immune system in the lung is particularly challenged with maintaining homeostasis. The alveolar surface, which is important for the gas exchange, is about 70-140m^2 in area. The lungs are filled with 10,000-20,000 liters of ambient air every day.[2] The inhaled air contains billions of particles, dust, allergens or other harmful pathogens. Hence, a network of specialized immune cells is required to clean the airways and maintain homeostasis. However, in the case of assault by pathogens, a powerful immune response has to be initiated. Among the first line of response are innate immune cells. Of these, mononuclear phagocytes (MNPs), which consist of macrophages, monocytes, and dendritic cells (DCs), are specialized in the uptake and clearance of the antigens. Additionally, the antigens can also be presented by MNPs to activate T cells, which are cells of the adaptive immune system. As MNPs line the respiratory mucosa, they act as sentinels of the pulmonary immune system and help to maintain steady state.

In the lungs, macrophages are the most abundant innate immune cells and are particularly powerful in the uptake of foreign particles. Alveolar macrophages are the first cells on the luminal side of the lungs to induce an immune response or to clear the particles "silently." Upon recognition of a pathogen or other danger signals by specialized pattern recognition receptors, macrophages release chemical mediators.[3] In case of a pathogen, those mediators can be cytokines to induce inflammation or chemokines to attract other immune cells to the site of antigen exposure.[4, 5] DCs are

another group of MNPs. DCs have specialized in activating naïve T cells.[6] For this, DCs take up antigen and migrate to the draining lymph nodes, where the antigen is presented to naïve T cells. Upon activation, T cells migrate back to the site of infection and clear the tissue from the antigen by releasing cytokines or by cytotoxicity mediated cell death.[7] To date, the knowledge about the innate and adaptive immune system has provided curative and preventive therapies. Yet, in the future, humanity will face new challenges with antibiotic resistant bacteria, novel emerging viruses and tumorous malignancies.[8-10] Thus, profound knowledge of immune cells during steady state conditions are required.

Sarcoidosis is a multi-systemic inflammatory disease primarily affecting the lungs that is often accompanied by an impaired quality of life. It is characterized by the presence of granulomas, which are multinucleated giant cells that contain an antigen on the inside to shield the surrounding tissue.[11] The etiology of sarcoidosis is still unknown. There are indications that sarcoidosis is of infectious origin, but an overreaction of the immune system against host structures has also been proposed.[12] MNPs are likely critical in the initiation of the immune response by encountering the antigen, that leads to the subsequent release of granuloma-forming cytokines, and the activation of T cells.[13] Hence, studying MNPs during steady state can pave the way to better understand the pathogenesis of sarcoidosis in order to clear disease and ultimately help patients to improve their quality of life.

This thesis focuses on studying mononuclear phagocytes, in the human respiratory tract during steady state and sarcoidosis. It provides an overview of the distribution, phenotype, and function of mononuclear phagocytes in healthy and diseased lungs. It shows the importance of studying MNPs as they contribute to inflammation in sarcoidosis and have a significant value as an outcome predictor.

2 AIMS OF THESIS

The overall aim of this thesis was to map the mononuclear phagocyte network in the human respiratory tract to better understand their function in healthy individuals and to decipher their importance during sarcoidosis.

The specific aims for the studies were as follows:

- To map the mononuclear phagocyte network in the respiratory tract and in blood during steady state (**Paper I**) and in sarcoidosis patients (**Paper II+III**),

- To identify unique transcriptional characteristics specific to blood and respiratory mononuclear phagocytes in sarcoidosis patients compared to steady state conditions (**Paper III**),

- To study the functional properties of blood and lung mononuclear phagocytes during steady state and in sarcoidosis patients (**Paper III+IV**),

- To investigate whether prevalence or function of mononuclear phagocytes at time of diagnosis associate with disease outcome in sarcoidosis patients (**Paper III**).

3 MONONUCLEAR PHAGOCYTES

Mononuclear phagocytes (MNPs) are a group of innate immune cells comprised of monocytes, macrophages, and dendritic cells (DCs).[14] MNPs surveil tissues for potential threats and help maintain homeostasis.[15] Common features of MNPs are their ability to sense and respond to potentially harmful pathogens by taking up foreign substances and producing cytokines.[16] Additionally, MNPs are professional antigen-presenting cells (APCs) that activate T cells in response to foreign antigens.[17] Besides overlapping features, each of the MNP subsets also have specialized functions.[18] In recent years, it has become evident that the MNP network is highly heterogeneous and its diversity has just started to unravel. Our increasing knowledge about the influence of the tissue microenvironment and inflammation on MNPs will improve the design of better vaccination strategies and therapeutics.[19, 20]

3.1 HISTORY OF MONONUCLEAR PHAGOCYTES

The first detailed knowledge of MNPs dates back to the 19th century.[19] Observations that splenic cells internalized red blood cells or contained bacilli indicated that specialized cells were able to take up particles.[20] However, at that time, it was unclear whether this was a beneficial process. On the contrary, it was assumed to be a process leading to the spread of bacteria.[21] Eventually, ground-breaking work by Ilya Metchnikov described the existence of cells actively taking up foreign particles, a process termed phagocytosis.[22] Also, Metchnikov considered phagocytosis a defense mechanism of the cells to protect the host. This theory gave rise to the concept of cellular innate immunity. Later, all known phagocytosing cells were described as constituting the reticuloendothelial system (RES) by Aschoff in 1924.[23] Depending on the tissue and person describing the phagocytes, up to 30 different names existed for similar cells.[24]

In the early 20th century, it became evident that macrophages arise from circulating monocytes.[25, 26] The pioneering work of Robin van Furth in the 1960s and 1970s distinguished monocytes and macrophages from polymorphonuclear phagocytes, known as granulocytes.[27, 28] Van Furth further concluded that tissue macrophages are constantly replenished by circulating monocytes that arise from a promonocyte in the bone marrow.[28] Due to the relationship between monocytes and macrophages and their differences to granulocytes, the term mononuclear phagocytes (MNPs) was established. In the 1970s, a “dendritic-shaped cell” similar, but with distinct functions, from macrophages was identified by Steinman and Cohn and called dendritic cells.[29, 30] Due to their similarity with monocytes and macrophages in morphology, origin and function, DCs became part of the MNP system.[31] Continued research in the past decades has revealed high heterogeneity among MNPs, in particular regarding their origin and function.

3.2 ONTOGENY OF MONONUCLEAR PHAGOCYTES

One way to classify MNPs is based on ontogeny - the studies of cellular origin.[14] The use of mouse models and adoptive transfer of precursor cells into irradiated hosts have identified distinct monocyte and DC precursors and deciphered the developmental origin of MNPs.

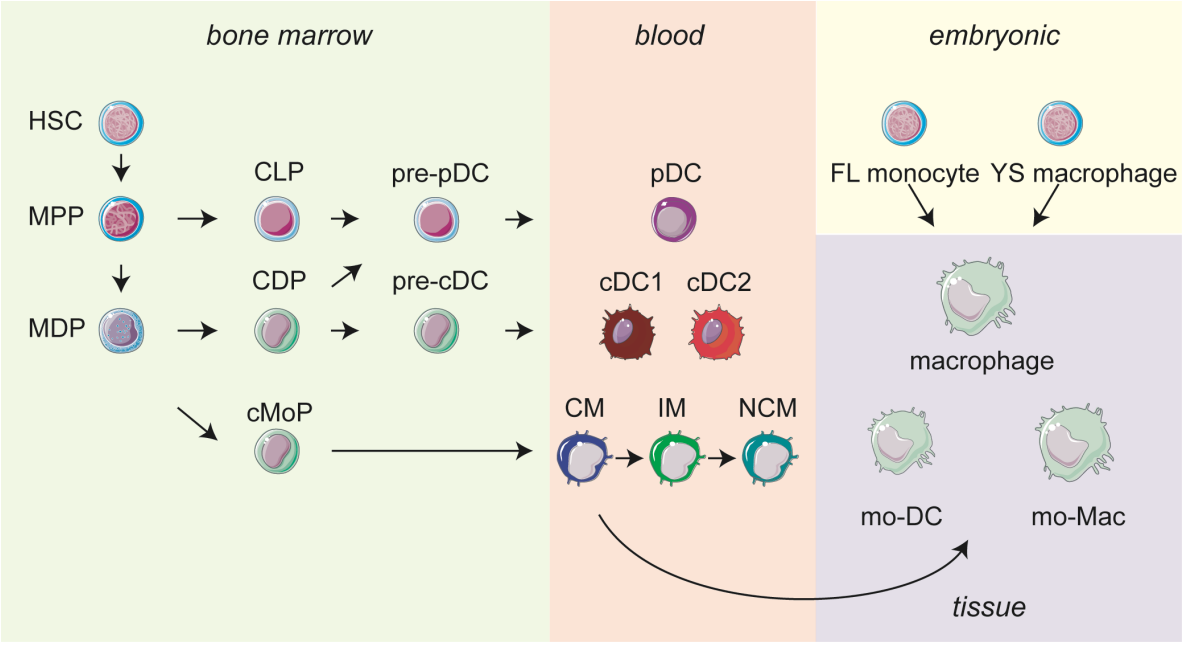

Figure 1. Hematopoiesis and development of MNPs. A simplified model with focus on the origin of MNPs. Hematopoietic stem cells (HSCs) differentiate into multipotent precursors (MPPs). MPPs give either rise to common lymphoid progenitors (CLPs) or monocyte/ macrophage–DC progenitors (MDPs). CLPs develop into pre-pDC precursors that can also arise from common DC precursors (CDPs), which develop from MDPs. Pre-pDCs give rise to pDCs. CDPs develop into pre-cDCs and finally to cDC1s and cDC2s. Classical monocytes (CMs) arise from common monocyte precursors (cMoPs) that develop from MDPs. In blood, CMs differentiate into intermediate monocytes (IMs) and non-classical monocytes (NCMs). CMs extravasate to become tissue monocytes or differentiate into monocyte-derived DCs (mo-DCs) or macrophages (mo-Macs). Tissue-resident macrophages are also seeded by independent precursors during embryogenesis from yolk sac (YS) macrophages or from fetal liver (FL) monocytes.

Historically, DCs and particularly macrophages were thought to be descendants of highly plastic monocytes. However, recent advances using lineage tracing, single-cell technologies, and cellular barcoding have identified individual precursors of the monocyte, DC, and macrophage cell lineages.[32] A major paradigm shift brough about by advanced technologies was the elucidation of the origin of tissue macrophages.[33] For decades, it was believed that monocytes differentiate into macrophages upon entering the tissue. This knowledge was challenged by the discovery of independent macrophage development from precursors in the embryonic yolk sac or the fetal liver. The distinct origin of tissue-resident macrophages has been shown for a variety of organs including the skin, brain, liver, and lungs.[34-37]

In detail, as illustrated in **Figure 1**, hematopoietic stem cells (HSCs) give rise to multipotent precursors (MPPs) followed by the common myeloid precursors (CMPs). In both mouse and human, DCs and monocytes diverge at the stage of the monocyte/macrophage–DC progenitors (MDPs) developing into the common DC precursors (CDPs) and the common monocyte precursors (cMoPs).[38-43] The cMoPs give rise to monocytes in the presence of colony stimulating factor 1 (CSF1).[44] Tissue-resident macrophages derive from several sources. During development, macrophages are seeded from the yolk sac as well as the fetal liver while in adults, macrophages either self-renew or differentiate from extravasated monocytes.[34] Crucial for the development of DCs is the expression of the Fms-like tyrosine kinase 3 (FLT3) and the presence of the growth factor FLT3 ligand.[45] From CDPs, pre-conventional DCs (pre-cDCs) and pre-plasmacytoid DCs (pre-pDCs) progenitors develop.[39] There is evidence that the two cDC subsets, characterized by expression of the transcription factors IRF8 and IRF4 and termed cDC1s and cDC2s, respectively, derive from distinct pre-cDC precursors.[46-48] pDCs develop from the pre-pDC precursors relying on the transcription factors E2-2 and ZEB2 for development.[49, 50] However, the precursor of pDCs is currently debated as it has also been shown that pDCs can develop from common lymphocyte progenitors (CLPs).[51] This suggests that pDCs may be of lymphoid origin in contrast to cDCs and monocytes, which are of myeloid origin.

3.3 MONONUCLEAR PHAGOCYTE SUBSETS

The MNP network *in vivo* is highly complex and our understanding of MNP subsets as well as their function is constantly updated. Importantly, most data were generated in animal models and translation into the human system has to be interpreted carefully. It has become obvious that relying merely on surface epitopes was not suitable for the comparison of MNP subsets between mice and humans or when comparing different tissues.[52] Also, monocytes are highly plastic and can acquire a macrophage or DC-like phenotype upon entering tissues, and can thereby share many surface epitopes with *bona fide* DCs and tissue-resident macrophages.[53] Hence, identification of transcription factors and transcriptomic signatures specific to MNP subsets were needed. Unbiased mass cytometry and single-cell technologies identified gene signatures specific to MNP subsets and their successors and allowed the careful mapping of MNP heterogeneity in tissues at steady state and during inflammation.[54-57] Common to identifying all MNP subsets is their surface expression of the human leukocyte antigen DR (HLA-DR). HLA-DR is expressed by all APCs including B cells. In human blood, three different monocytes can be distinguished by their expression of CD14 and CD16.[58] Further pDCs can be further identified by the expression of the IL-3 receptor (CD123) as well as cDC1s and cDC2s by their expression of CD141 and CD1c, respectively **(Figure 2A)**.[59]

MNPs are equipped with tools to degrade external antigens. Macrophages have herein specialized compared to monocytes and DCs.[60, 61] A further common feature to all MNPs is their expression of pattern recognition receptors (PRRs) that sense the foreign materials and subsequently lead to the production of cytokines.[53, 62, 63] Monocytes, in particular, express a wide range of PRRs, which allows them to respond rapidly and effectively upon encounter of an antigen.[64] Finally, MNPs have the ability to activate T cells by loading antigenic peptides on MHC class I and II molecules and presenting them to cognate T cell receptors. Among MNPs, DCs have specialized in activating naïve T cells, which takes place in the draining lymph nodes. DC upregulate receptors that mediate migration to the lymph node upon encounter of an antigen.[65, 66] After arrival in the lymph node, DCs have matured by upregulation of co-stimulatory receptors and subsequently instruct naïve T cells.[16] Besides their overlapping functions, each individual MNP subset has also distinct functional features **(Figure 2B)**. Together, MNPs help maintain homeostasis and respond effectively to potential threats. A summary of surface epitopes and specialized tasks of MNP subsets is summarized in **Table 1**.

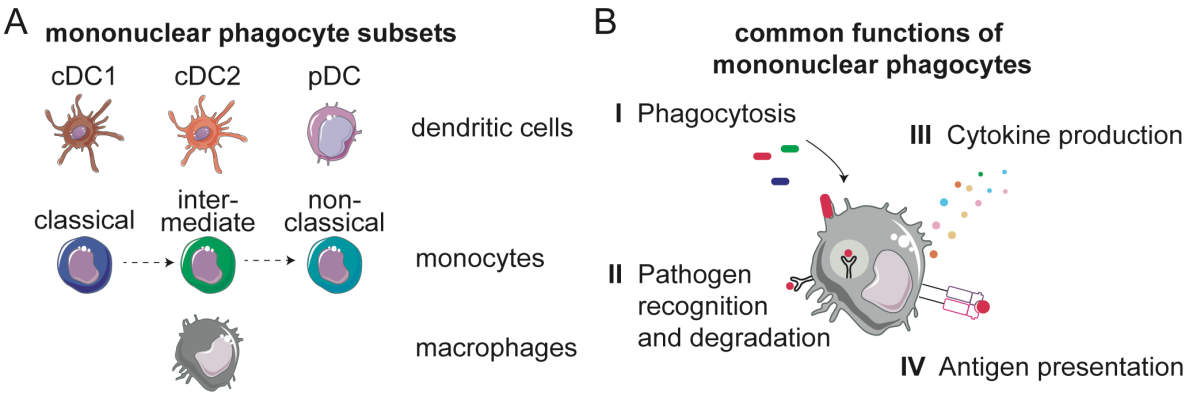

Figure 2. Mononuclear phagocyte subsets and common functions. (A) Human mononuclear phagocytes consist of DC and monocyte subsets as well as macrophages. **(B)** Common functions shared by all MNPs. **(I)** The uptake of foreign antigens (phagocytosis), **(II)** the recognition by PRRs and degradation of antigens. MNPs respond to PRR activation by **(III)** secretion of cytokines and **(IV)** presentation of antigens on MHC molecules to T cells.

3.3.1 Monocytes and macrophages

The role of monocytes has for a long time focused on their ability to differentiate into tissue-resident macrophages or monocyte-derived DCs. Only recently, the importance of monocytes as an individual MNP subset was acknowledged with their role as first responders to inflammation.[67] Monocytes respond rapidly by producing proinflammatory cytokines upon stimulation.[68] They account for about 10 % of circulating leukocytes and have a high turnover rate.[69] In the bone marrow (BM), cMoPs are highly proliferative and post-mitotic monocytes are retained creating a pool of monocytes.[70] During inflammatory conditions, BM monocytes are released and migrate quickly to the site of inflammation.[71-73] In humans, three monocyte subsets

have been identified in circulation: classical monocytes (CMs), intermediate monocytes (IMs), and non-classical monocytes (NCMs).[18] Monocytes are characterized by their expression of the lipopolysaccharide (LPS) co-receptor (CD14) and the Fc-gamma receptor III (CD16). CMs are $CD14^{+}CD16^{-}$, IMs express high levels of both CD14 and CD16, and NCMs are CD14 low and $CD16^{+}$. This differential expression of identifying markers already indicates the developmental progress from CMs to IMs and NCMs. In the BM, only CMs develop from the cMoPs and express high levels of CCR2, a chemokine receptor critical for egress from the BM.[74] Once released into the circulation, CMs remain in circulation for about one day.[70] About 1% of CMs differentiate into IMs while 99% of CMs extravasate into the tissues.[70] CCR2 is believed to also mediate extravasation of CMs. As IMs express CCR2 at lower levels compared to CMs, IMs remain in circulation and ultimately differentiate into NCMs with an average half-life time of seven days (**Figure 1**).[70, 75]

A clear distinction between the monocyte subsets is difficult as the differentiation from CMs to IMs and NCMs is smooth and characterized by the up- and downregulation of cell surface receptors.[75] Another challenge in the study of monocytes is that an intermediate population is missing in mice, where monocytes are characterized based on their expression of Ly6C.[76] Functionally, monocytes are potent in producing both pro- and anti-inflammatory cytokines.[68] This feature is mostly attributed to classical and intermediate monocytes, although NCMs were also shown to produce pro-inflammatory tumor necrosis factor (TNF) upon encountering viruses.[77] Additionally, NCMs are potent in maintaining epithelial cell integrity during atherosclerosis.[78]

Upon extravasation, monocytes are exposed to the local cytokine environment that differs between steady state and inflammation. Due to their plasticity, monocytes acquire a phenotype based on the cytokine milieu. A fraction of monocytes remain as tissue monocytes and are distinct from monocyte-derived DCs (mo-DCs) and monocyte-derived macrophages (mo-Macs).[53]

In 2000, the model of M1 and M2 macrophages was introduced as an approach to describe pro- and anti-inflammatory macrophages.[79] However, in recent years it has become more obvious that the heterogeneity and dynamics of macrophages cannot be explained with the simple M1/M2 dichotomy. Extensive research on tissue-resident macrophages has led to the characterization of key points that shape macrophage identity.[80] First, the origin is a key factor distinguishing macrophages seeded during embryogenesis versus mo-Macs. During steady state, tissue-resident macrophages arise from three waves to seed various tissues: first, from the yolk sac followed by fetal liver monocytes, and during adulthood from circulating monocytes.[33, 35-37, 80-82] Second, their location and time spent within a tissue is of importance as proposed in the *niche theory.*[34, 83] Within each tissue, macrophages are engrafted in niches formed by tissue epithelial cells that produce growth factors required for macrophage identity. The phenotype and function of macrophages are dependent on the local environment,

which can also differ between niches in one organ. Finally, inflammation shapes and influences macrophages.[84] During steady state and inflammation, macrophages continuously undergo cell death as well as self-renewal.[85-90] Empty niches due to infection or inflammation are preliminarily filled with recruited monocytes that mimic the function of tissue-resident macrophages before replenished with tissue-resident macrophages. Altogether, the macrophage compartment is very heterogeneous, and macrophages should cautiously be described in their respective tissue and inflammatory context.

3.3.2 Dendritic cells

DCs are often described as the cells that bridge the innate and adaptive immune systems, particularly as they are the most potent cell subset at activating naïve T cells.[91-93] Human DCs can broadly be divided into three distinct populations: plasmacytoid DCs (pDCs) and two myeloid or conventional DCs (cDCs). The expression of surface markers on DCs in human and mice differ vastly.[32] Hence, it was difficult for a long time to translate findings across species. In a recent study however, common surface epitopes and transcription factors were describe to reliably identify pDCs and cDCs across species and tissues.[54] Furthermore, recent advances using single-cell technologies have identified further non-canonical DC subsets suggesting more heterogeneity already at steady state.[94-96]

pDCs, identified based on the expression of the IL-3 receptor (CD123), are most commonly found in circulation and lymphoid tissue.[97-99] pDCs have high expression of Toll like receptors (TLRs) 7 and 9, which sense viral single-stranded RNA and double-stranded DNA, respectively.[100] Upon TLR activation, pDCs release high amounts of antiviral interferon alpha (IFNα).[100, 101] Despite their potent antiviral activity, pDCs are poor T cell activators.[102, 103] These observations in context with the debated myeloid or lymphoid progenitor of pDCs has led to the assumption that pDCs may be contaminated with cDC precursors resulting in one innate lymphoid population and one cDC precursor population.[51, 94, 95]

In human blood, cDC1s represent only a small fraction of DCs (about 1/10 of cDC2s) and are characterized by the expression of CD141.[59] As CD141 is also expressed by other cells in tissue, using X-C motif chemokine receptor 1 (XCR1) or the transcription factor interferon regulatory factor 8 (IRF8) were shown to be useful to reliably identify cDC1s.[54, 104] However, using transcription factor staining is not always possible, such as in experiments using fluorescence-activated cell sorting (FACS) and subsequent functional experiments. Functionally, cDC1s were shown to be superior in cross-presentation, a process characterized by the uptake of apoptotic cells and presentation of antigen peptides to $CD8^+$ T cells.[105-107] However, these data could not be confirmed in humans as cDC2s and mo-DCs also cross-present antigen to $CD8^+$

T cells.[108-111] Furthermore, cDC1s were better at mediating Th1 responses compared to cDC2s by high expression of IL-12.[105, 107] In humans, CD4$^+$ T cell activation is less restricted to cDC1s as cDC2s and mo-DCs were also shown to be potent Th1 inducers.[112, 113]

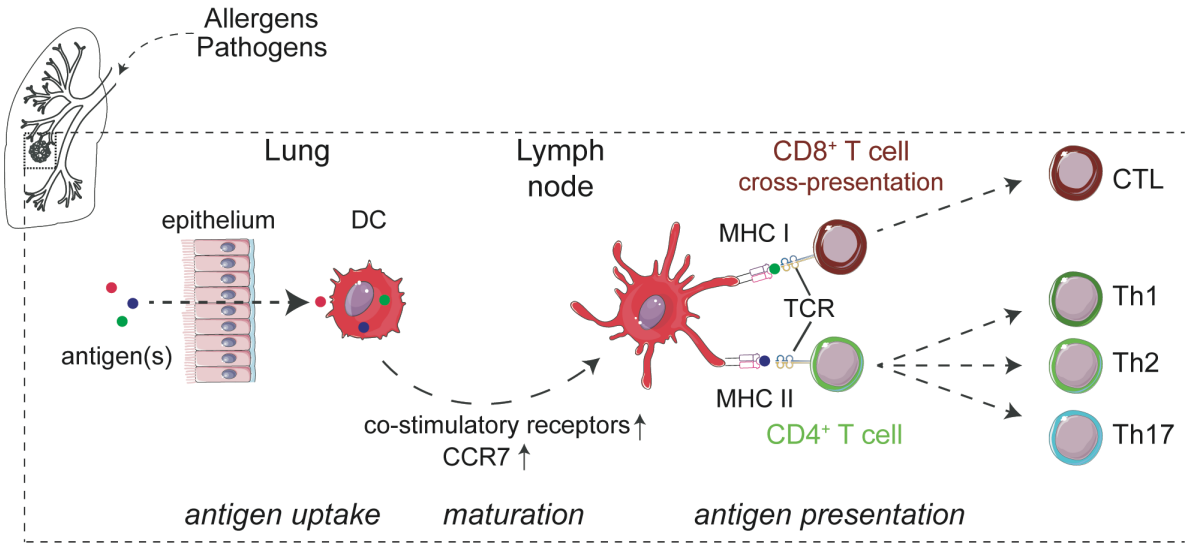

Figure 3. Overview of T cell activation by dendritic cells. Allergens or pathogens are inhaled and cross the epithelium where they are taken up by MNPs, such as DCs. DCs recognize antigenic structures and mature by upregulating co-stimulatory molecules and CCR7, which mediates trafficking to the draining lymph nodes. In the lymph nodes, DCs present the antigen on MHC I or MHC II to CD8$^+$ cytotoxic T lymphocytes (CTL), also called cross-presentation, and CD4$^+$ T cells, respectively. Depending on the cytokine signals by the DCs, CD4$^+$ T cells differentiate into specific T helper cell (Th) subsets.

The most frequent myeloid DC subset in human blood are cDC2s. cDC2s are characterized by their expression of CD1c.[59] CD1c is also expressed by mo-DCs in tissues.[55, 114-116] Hence, further markers such as SIRPα (CD172) or the transcription factor interferon regulatory factor 4 (IRF4) are used to specifically identify cDC2s.[6, 54] In comparison to cDC1s, cDC2s express a broader variety of TLRs, which improves their capability to sense foreign antigens.[117] cDC2s were shown to activate naïve T cells and skew them towards Th1, Th2, Th17 as well as regulatory T cells (Tregs) by secreting a variety of cytokines including TNF, IL-1β, IL-10 and IL-23.[113, 118-120] This diversity in potential cytokine responses suggests a heterogeneity in cDC2s. The migration of cDC2s to the lymph node is mediated by CCR7, CCL19, and CCL21.[121, 122] The upregulation of CCR7 as well as co-stimulatory molecules CD80 and CD86 are together called maturation. Upon arrival in the lymph node, DCs have processed the antigen and loaded on MHC class I and II molecules to activate naïve CD8$^+$ and CD4$^+$ T cells, respectively **(Figure 3)**.

3.3.3 Monocyte-derived cells

Monocyte-derived macrophages (mo-Macs) or dendritic cells (mo-DCs) were for a long time used as a model to study the function of macrophages and DCs *in vitro*. Using monocytes from the blood and adding M-CSF or GM-CSF leads to the differentiation into mo-Macs,[123, 124] while GM-CSF and IL-4 generates mo-DCs.[125] Those *in vitro* model systems are helpful to study the basic functions of mo-Macs and mo-DCs as high numbers of cells can be generated in a short time. However, with the advanced knowledge of MNP origin, it has become obvious that *in vitro*-generated monocyte-derived cells are also scrutinized as they are not a reflection of *bona fide* DCs or tissue-resident macrophages.[126]

Table 1. Characteristics of human mononuclear phagocytes.[54, 55, 114, 115, 127-129]

Human MNPs	cDC1	cDC2	Monocytes	Macro-phages	mo-DCs
Phenotype	CD141, XCR1, CLEC9A, IRF8	CD1c, CD172a, IRF4	CD14, CD16, CCR2	CD11b, CD16, CD206, CD163	CD14, CD1a-c, CD172a, CD206
Cytokines produced	TNF, IL-6, IL-12	TNF, IL-1β, IL-6, IL-12, TGF-β, IL-23	TNF, IL-6, IL-12, IL-10, TGF-β	TNF, IL-1β, IL-6, TGF-β	TNF, IL-1β, IL-6, IL-12, IL-23
T cell activation	CD4 (Th1) CD8	CD4 (Th2, Th17) CD8	-	-	CD4 (Th1, Th17) CD8

However, monocyte-derived cells have also been found *in vivo*, primarily contributing to inflammatory conditions through their production of TNF, IL-6 or IL-1β.[116] Depending on the observations, these cells were named inflammatory monocytes/DCs[116] or TipDCs[130] in human and mouse inflammation models, respectively. However, human mo-DCs also develop during steady state upon entering tissues and are characterized by their common expression of monocyte and DC markers such as CD14 and CD1c, respectively.[55, 114, 115, 128, 129, 131-133] Additionally, mo-DCs express HLA-DR and CD11c together with the tissue-specific mannose receptor (CD206) but lack the macrophage specific marker CD163.[111, 114, 115] Besides their rapid recruitment to the site of inflammation and production of pro-inflammatory cytokines, mo-DCs were also shown to activate $CD4^+$ and $CD8^+$ T cells.[116, 133] Upon activation, mo-DCs also secrete IL-23, a potent cytokine that induces Th17 polarization. These observations derive from

ascites and rheumatoid arthritis models with pathogenic Th17 cell involvement (**Table 1**).[116, 133] Hence, mo-DCs may contribute to disease severity during inflammatory disease.

In conclusion, comparative analysis of mice and humans have characterized DCs, monocytes, and macrophages with distinct cell surface and transcription factor expression allowing for the comparison of MNPs across species. However, the challenge remains to map in detail the distribution and function of human MNPs in different tissues.

4 THE RESPIRATORY SYSTEM

The respiratory system is divided into an upper (nasal cavity, pharynx, and larynx) and lower respiratory tract (trachea, bronchi, and lungs). In contrast to mice, sampling the human lower respiratory tract is challenging and often an invasive procedure. Studying pulmonary immune cells is often accompanied by caveats.

4.1 SAMPLING THE RESPIRATORY TRACT

Surgical lung resections are often performed on patients with lung cancer or pulmonary fibrosis.[134] In order to compare immune cell composition and function, often cells from the affected (disease) and non-affected (control) regions of the lungs are obtained. However, it is unclear to what extent an unaffected area from a patient with pulmonary disease resembles the lung tissue of a healthy individual. Often an ongoing inflammation alters neighboring areas of the organ thereby also affecting immune cells.[83] Additionally, excised tissue contains blood vessels with circulating immune cells that could be confused with tissue-resident cells.[115] As an advantage of surgical resections, samples from the lymph nodes draining the diseased and non-diseased areas of the lungs can often be obtained.[135]

Bronchoscopy is another invasive but safe and well-tolerated method to sample the respiratory tract. An overview of the sampling sites feasible by bronchoscopy is illustrated in **Figure 4**. A fiberoptic bronchoscope allows for the visualization and sampling of the mucosal surfaces of the trachea, bronchi, and alveoli.[136] During a bronchoscopy, several methods sample the lower airways. From proximal to distal: collection of endobronchial biopsies (EBB), bronchial brush (BB), bronchial wash (BW), as well as bronchoalveolar lavage (BAL).[114, 137] While EBBs collect bronchial tissue specimen, lavages are performed with a saline solution that collects cells lining the mucosal surface. To what extent cells collected by lavages reflect lung tissue is currently debated.[138, 139] An advantage of the lavages is the subsequent minimal manipulation of cells as compared to enzymatic digestion of bronchial tissue specimens obtained by EBBs. Enzymatic treatment can cleave surface proteins that are required for the identification or phenotyping of immune cells.[140, 141] Also, tissue digestion often requires incubation at 37°C for optimal enzyme function, which metabolically affects cells. During a bronchoscopy, also the mediastinal and hilar lymph nodes can be sampled using endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA).[142] In the clinical diagnostics, EBUS-TBNA has proven useful as an on-site diagnostics exam.[143] Sampling healthy volunteers, however, is difficult as only inflamed lymph nodes are usually large enough for optimal sampling.

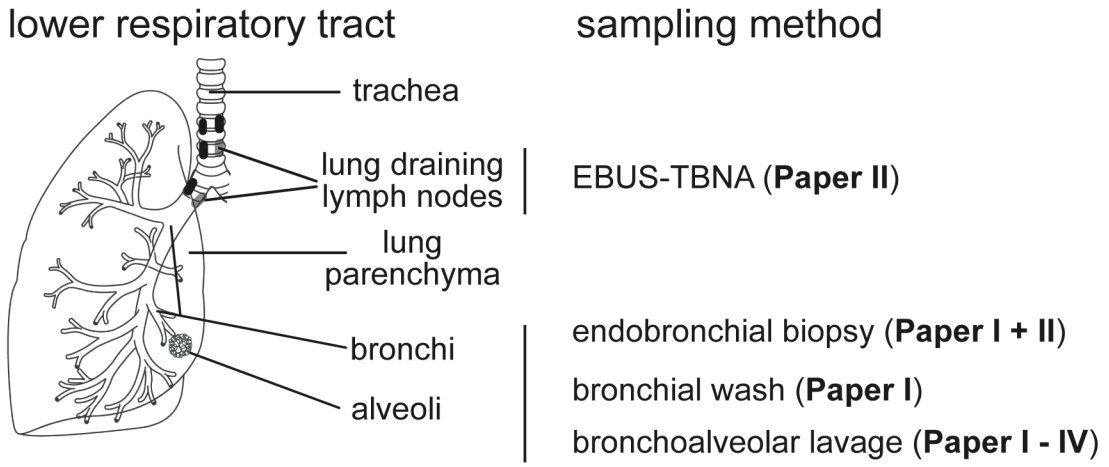

Figure 4. The lower respiratory tract and sampling sites used in this thesis. The lower respiratory tract consists of trachea, bronchi and the lung parenchyma. Bronchoscopies were performed to sample the different sites of the lower respiratory tract. EBUS-TBNA: endobronchial ultrasound-guided transbronchial needle aspiration.

Another factor to consider when processing samples for research is time. After retrieval of the tissue samples, it is critical to provide low temperatures to not induce metabolic changes in the cells that would result in the up- or downregulation of proteins. Depending on the research question, samples can either be processed and used for experiments immediately or cells frozen for use at a later date. However, one cycle of freeze-thawing can also have effects on cell functionality and surface protein expression.[114] Hence, it is important to employ consistent strategies within a project.

In conclusion, sampling the human respiratory tract is often difficult, and this has limited our knowledge on the immune system in this compartment. Hence, studies sampling different anatomical sites of the respiratory tract side-by-side are needed. This will improve our understanding on how immune cells are distributed and function along the respiratory tract, and in comparison, to their well-known blood counterparts.

4.2 PULMONARY MONONUCLEAR PHAGOCYTES

Lung diseases are one of the leading causes of deaths in the world.[144] Often the immune system can handle short-term exposure to noxious particles, but long-term exposure may lead to irreversible changes, including fibrosis.[145] Common pulmonary diseases include tumorous malignancies, infections (e.g. tuberculosis), chronic obstructive pulmonary disease (COPD), asthma and, less frequently, interstitial diseases (e.g. idiopathic pulmonary fibrosis, sarcoidosis). In order to better understand the contribution of MNPs to disease pathogenesis, their role during healthy steady state conditions has to be investigated.

During the past years, we and others have used different approaches to map human MNPs from the respiratory tract during steady state.[114, 115, 129] MNPs from lavages, bronchial biopsies, as well as lung tissues and their draining lymph nodes from deceased donors showed the presence of macrophages, monocytes, and DCs. The frequency of the MNP subsets varied between the studies due to the strategy used to define them.[114, 115, 129] Interestingly, most pronounced differences in cell composition were observed between blood and the respiratory tract. A shift towards intermediate monocytes, increased percentage of cDC1s and very few pDCs were observed in the lungs compared to blood.[114]

Functionally, pulmonary MNPs differ in their abilities to respond to challenges. For example, while all MNPs have been shown to phagocytose bacterial particles, macrophages and monocytes especially excel at this.[129] Furthermore, monocytes potently produce TNF upon TLR stimulation, while DCs were better in activating allogeneic T cells than monocytes.[114, 115] These data highlight the heterogeneity of MNPs within the respiratory compartment and a distinct division of labor between the subsets in maintaining homeostasis.

While the role of MNPs during pulmonary inflammation and infection has been studied for many years, observations have often contradicted each other or have been inconclusive. This has likely been due to the strategy to identify MNPs and a small sample size.[146] Among the most common particles that the pulmonary innate immune system has to tackle are caused by cigarette smoke. Smoking is a risk factor for several pulmonary diseases such as COPD or lung cancer.[147, 148] Alveolar macrophages are affected by smoking by increasing cell numbers and altered functions.[149-153] The constant exposure to cigarette smoke also leads to persistent inflammation in the lungs affecting other MNP subsets. DCs accumulate and are more mature in the lungs of COPD patients compared to healthy controls.[154] However, there are also reports implying that smoking COPD patients that have less mature or tolerogenic DCs compared to smokers without COPD, which may be caused by different isolation and analysis methods.[155, 156]

In general, studies with patients suffering from pulmonary diseases often show a heterogenous picture. Patients may wait months or years before seeking health care and present at the clinic at different stages of the disease. Consequently, analysis of immune parameters at that time may only be a snapshot of the disease. Thus, a careful clinical characterization accompanied by a close follow up of patients longitudinally is needed to better understand immune mechanisms involved during the disease course.

5 SARCOIDOSIS

Sarcoidosis is a systemic inflammatory disease characterized by the formation of non-caseating granulomas in affected organs. Granulomas can occur in the skin, eyes, heart, and the central nervous system, but the most commonly affected organs are the lungs and the lung-draining lymph nodes.[11, 157] As granulomas can form in multiple organs, patients often present with a multitude of different clinical symptoms. It has been estimated that 30-40% of patients usually recover from sarcoidosis within two years and 80% recover within five years.[158-161] However, 20% develop chronic progressive disease that ultimately leads to fibrosis and organ failure.[162] The clinical phenotype is likely determined by genetics and influenced by the environment, however, these factors do not fully explain sarcoidosis pathogenesis.[163] Therefore, studying the underlying immunological mechanisms is a central aspect of sarcoidosis research to better understand which factors determine the course of the disease.

5.1 EPIDEMIOLOGY AND CLINICAL CHARACTERISTICS

Sarcoidosis has been reported around the world but with differences based on the geographical region and ethnicity.[164] The highest incidence and prevalence of sarcoidosis are found in the Scandinavian countries and among African Americans.[159, 164-166] In Sweden, the incidence is 11.5 per 100,000 individuals per year, which equals to about 1200 new cases each year.[167] Sarcoidosis can affect both men and women.[164] The epidemiological data indicate differences in the onset of sarcoidosis. In men, the highest incidence is between 30 and 50 years of age, whereas women have the highest incidence rates between the age of 30-40 and a second peak between 50 to 60 years.[167]

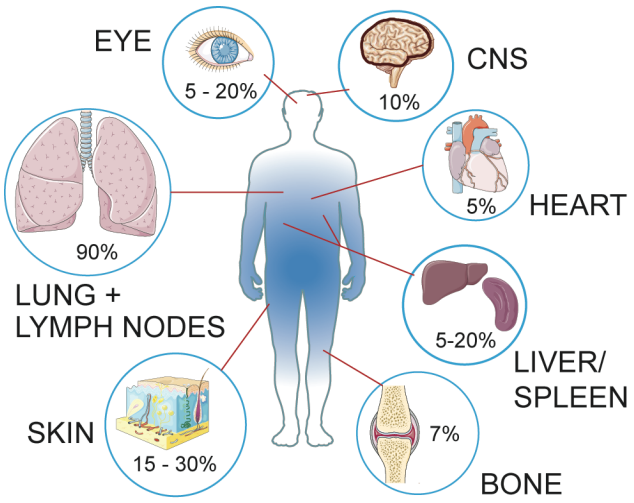

Figure 5. Organ involvement in sarcoidosis. Shown are the most common tissues affected by granuloma formation. Percentages show the prevalence of organ involvement.

The diagnosis of sarcoidosis is based on findings of non-caseating epithelioid cell granulomas in affected organs. Malignancies, infections and other granuloma-causing antigens should be excluded prior to sarcoidosis diagnosis.[168] The range of clinical symptoms depends mostly on the organs involved (**Figure 5**).[11] A common, not organ-specific symptom is fatigue, which greatly affects the quality of life in patients.[169] In pulmonary sarcoidosis, organ-specific symptoms include dry cough or shortness of breath.[11] Intrathoracic lymph node enlargement and parenchymal infiltrates can be detected using radiographic tools like conventional X-ray and computed tomography scan (**Table 2**).[170, 171] To detect granulomas, patients undergo a bronchoscopy to sample biopsies from the bronchial mucosa. Additionally, lung parenchyma specimen can be obtained by transbronchial lung biopsies, and EBUS-TBNA can sample the lung-draining lymph nodes.[172] Additionally, a lavage of the lower airways is commonly performed to collect the bronchoalveolar lavage (BAL) fluid of the lower airways in order to retrieve the cells lining the bronchial mucosa and alveoli. Among BAL cells, an elevated ratio (>3.5) of $CD4^+$ to $CD8^+$ T cells serves as a diagnostic tool.[173]

Table 2. Chest radiographic staging according to Scadding.[172]

Chest radiography stage	Characteristics
0	No alterations
I	Bilateral hilar lymphadenopathy
II	Bilateral hilar lymphadenopathy with pulmonary infiltrates
III	Pulmonary infiltrates only
IV	Fibrosis, volume reduction

One third of sarcoidosis patients in Sweden present with an acute disease onset characterized by fever, enlarged bilateral hilar lymph nodes, erythema nodosum and/or periarticular inflammation.[174] This syndrome was first described by the Swedish physician Sven Löfgren in 1946 and later termed Löfgren's syndrome (LS).[175] About 76% of LS sarcoidosis patients resolve the disease within two years.[161] Most LS patients express the human leukocyte antigen (HLA)-DR with the subtype B1*03. In this cohort of patients, a spontaneous remission was observed in >95% of the cases implying a genetic and immune component contributing to disease outcome.[160, 161, 176] Hence, LS patients usually have a better prognosis compared to non-LS patients. Besides genetics, also immune cells may also play a role in the pathogenesis and resolution of LS and non-LS sarcoidosis.

Treatment of sarcoidosis focuses on relieving symptoms and preventing irreversible sequelae. No drug has been convincingly proven to change the disease course and outcome. Corticosteroids are the primary choice of medication in sarcoidosis patients. It can be combined with immunosuppressive drugs such as methotrexate and azathioprine as a second line treatment.[177] In refractory cases with progressive disease, anti-inflammatory biological drugs such as tumor necrosis factor (TNF) inhibitors may be used as a third line treatment.[178] However, studying the underlying immunological mechanisms that contribute to pathogenesis may identify patients in need of treatment early on.

5.2 ETIOLOGY AND PATHOGENESIS

Pulmonary sarcoidosis is characterized as a T cell-driven disease due to the high number of T cells accumulating in the lungs, particularly around the granulomas. The granuloma-causing antigen(s) have not yet been identified. The innate immune system, however, mounts an immune response against the antigen by release of pro-inflammatory cytokines and T cell activation. In the following section, details about the current understanding of the causative antigen as well as the immune response will be discussed.

5.2.1 The granuloma

Granulomatous structures, as formed by infection (e.g. tuberculosis) or as a response to foreign particles (e.g. beryllium), constitute a defense mechanism by the immune system to protect the tissue.[179] In general, the core of a granuloma consists of macrophages or epithelioid giant cells that have phagocytosed an antigen.[13] Depending on the antigen, macrophages release pro-inflammatory cytokines to eliminate the antigen and to recruit additional phagocytes. In sarcoidosis, it is hypothesized that the immediate response fails to clear the antigen. When the inflammation persists, macrophages aggregate and divide incompletely, forming multinucleated giant cells.[180] This process is mediated by the mTOR pathway.[181] The aggregation of macrophages is called a granuloma and can differ based on the antigen. In contrast to tuberculosis granulomas where bacteria induce necrosis in macrophages, sarcoid granulomas are non-necrotizing, indicating an agent with no mycobacterial background.[182, 183]

5.2.2 The quest for the antigen

The search for the sarcoidosis-inducing antigen has been ongoing for decades. However, a common antigen found in all sarcoidosis patients is still lacking. Several agents have been proposed. Bacterial DNA and proteins from *Mycobacteria spp* and *Cutibacteria spp* were detected in the granulomas of a cohort of sarcoidosis patients.[184] Additionally, T cells challenged with mycobacterial antigens such as the 6 kDa early secretory antigenic target (ESAT-6) or mycobacterial catalase-peroxidase (mKatG) showed enhanced activation and cytokine release, suggesting previous encounter.[185-187] Furthermore, immune responses were also detected after stimulation with self-antigens against vimentin, particularly in HLA-DRB1*03-positive LS patients.[188] Also, within the granulomas high levels of serum amyloid A (SAA) were found. SAA is an acute phase protein and is elevated during sarcoidosis. In contrast to other proposed antigens, SAA is likely not the causative agent but misfolded and aggregated protein stimulates TLR2 in macrophages.[189] Constant TLR2 stimulation leads to the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway and release of proinflammatory cytokines that promote granuloma formation.

It is also possible that genetic factors influence the formation of granulomas in sarcoidosis. As mentioned above, the HLA-DRB1*03 haplotype is commonly found in patients with Löfgren's syndrome. Furthermore, a clonal expansion of T cells with the variable alpha chain 2.3 of the T cell receptor (TRAV2.3) was identified in BAL of particularly LS patients.[190, 191] Increased number of TRAV2.3-expressing T cells associates with good prognosis.[192] This suggests the presence of a specific antigen being presented by a certain HLA molecule that is recognized by T cells expressing the specific T cell receptor TRAV2.3.

The search for the antigen(s) causing sarcoidosis continues as none of the above candidate agents were causative in all patients. It is also speculated that LS and non-LS sarcoidosis are separate disease entities caused by different agents.[12]

5.2.3 T cells in sarcoidosis

Patients with pulmonary sarcoidosis have an expansion of particularly $CD4^+$ T cells.[173] Activated T cells show mostly a T helper cell 1 (Th1) phenotype due to release of high levels of interferon gamma (IFNγ) after stimulation with candidate antigens *in vitro*.[193-195] Most T cells obtained from BAL expressed the transcription factor Tbet, which is specific for the Th1 lineage. The Th1 dogma was later challenged as also other cytokines such as IL-17 were found in the BAL fluid of sarcoidosis patients suggesting Th17 involvement.[196-200] T cells were also found to co-express Tbet and RORγt, the transcription factor for Th17 cells, suggesting plasticity in the T cell compartment.[197] The percentage of $Tbet^+RORγt^+$ cells was particularly high in BAL T cells from LS

patients compared to non-LS patients. T cells from LS patients also expressed higher levels of IL-17, which may influence the better outcome of LS patients compared to non-LS patients.[197] In contrast, another study identified $CXCR3^+CCR6^+$ T cells in BAL of sarcoidosis patients. Both are chemokine receptors specific to Th1 and Th17 cells, respectively. This T cell subset was termed Th17.1 cells.[198] Th17.1 cells were more abundant than Th1 cells in sarcoidosis patients and potently produced IFNγ upon stimulation.[198] From a clinical perspective, Th17.1 cells were expanded in BAL and lymph nodes of sarcoidosis patients and increased numbers correlated with progressive disease development.[199] These results contradict each other to some extent as $ROR\gamma t^+$ cells were expanded in LS patients, which usually resolve disease within two years, while Th17.1 cells were instead predicting disease progression.[200] An overview of T cells involved in sarcoidosis is shown in **Figure 6**. A combination of transcription factor, chemokine receptor, and cytokine staining in one panel could possibly help to identify the small but important differences between T cell subsets.

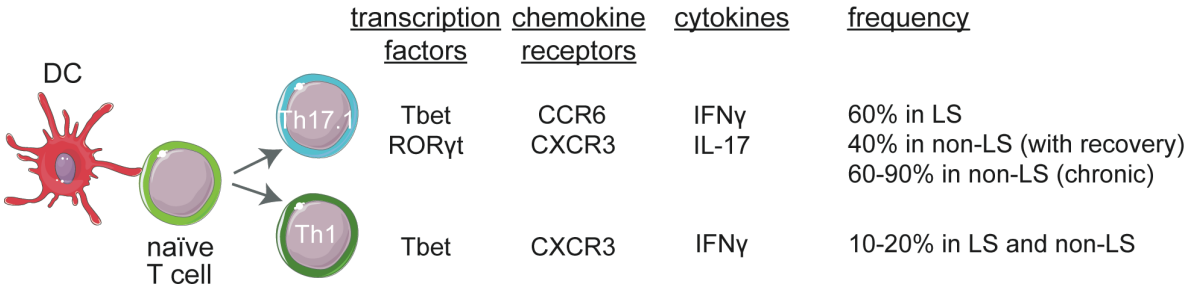

Figure 6. The phenotype of T cells in sarcoidosis. In sarcoidosis, naïve T cells become activated by DCs and differentiate mostly into Th1 or Th17.1 cells. Th17.1 cells are characterized by their expression of Tbet and RORγt and their chemokine receptors CCR6 and CXCR3. Th17.1 cells potently produce IFNγ and tor some extent IL-17. Despite their expansion in patients with chronic sarcoidosis, Th17.1 cells are also found in high numbers in LS patients that usually have a good prognosis.

These data highlight the heterogeneity of T cells in LS and non-LS sarcoidosis. Thus, it is important to understand how professional antigen-presenting cells (APCs) activate and skew the T cell response. That knowledge may help to target and manipulate APCs therapeutically in order to improve the resolution of the disease.

5.2.4 Mononuclear phagocytes in sarcoidosis

Alveolar macrophages (AMs) in sarcoidosis are well studied due to their abundance in the airways and their easy retrieval with BAL. AMs make up 95% of BAL cells in healthy controls and about 70-80% in sarcoidosis patients.[201, 202] AMs are potent in phagocytosing foreign particles and recognize antigenic structures with a specialized set of pattern recognition receptors.[114, 129] Upon recognition, AMs secrete soluble mediators to induce inflammation and to recruit cells to the site of antigen encounter.

In sarcoidosis, AMs are potent producers of TNF, IL-1β and IL-6 compared to macrophages from healthy controls upon stimulation with the TLR2 ligands and microbial candidate antigens ESAT-6, mKatG or SAA.[186, 189, 203] The role of AMs in activating T cells is less well studied. Early reports suggested that AMs from sarcoidosis patients are better at activating autologous T cells compared to macrophages from healthy controls.[204, 205]

A special role in sarcoidosis is attributed to TNF. AMs from sarcoidosis patients produce TNF intrinsically without stimulation.[206-208] TNF is important in forming and maintaining the sarcoid granulomas and high TNF production associates with disease severity in sarcoidosis.[209] Further, anti-TNF treatment was shown to be beneficial for sarcoidosis patients by improving their lung function or having less infiltrates on the chest x-ray.[178, 210-213] These data suggest that AMs in sarcoidosis are highly activated and contribute to inflammation, granuloma formation and maintenance as well as T cell activation.

Less well studied in sarcoidosis are pulmonary monocytes and monocyte-derived cells due to their resemblance to macrophages. We have recently described how MNPs, including pulmonary monocytes, are distributed in the respiratory tract and the lung-draining lymph nodes (LLN) of LS and non-LS sarcoidosis patients.[214] In blood, the frequencies of intermediate monocytes have been shown to be expanded in sarcoidosis patients, likely as a response to the systemic inflammation.[215-217] Functionally, monocytes produced less IL-10 upon stimulation compared to controls and induced fewer suppressive T cells.[218] Further studies have shown that blood monocytes were potent producers of TNF and IL-6 upon stimulation compared with controls, which could be due to higher expression of TLRs in sarcoidosis monocytes.[203, 215] Furthermore, monocytes from sarcoidosis patients also produced more IL-1β upon LPS stimulation, indicating activation of the NLRP3 inflammasome pathway that may be involved in granuloma formation.[219] Collectively, these studies indicate a pro-inflammatory phenotype for circulatory monocytes in sarcoidosis.

Dendritic cells (DCs) may play a crucial role in sarcoidosis as the superior subset to activate T cells. However, recent reports were inconclusive about the contribution of DCs to pathogenesis in sarcoidosis Overall, frequencies and numbers of DCs were unchanged or only slightly increased in blood and BAL of sarcoidosis patients compared to healthy controls.[220, 221] DCs found surrounding granulomas were mature as assessed by CD86 expression.[221] In a different report, BAL DCs were less mature compared to controls as assessed by CD1a expression.[220] This heterogeneity in distribution and maturation was also reflected in their capacity to induce T cell proliferation. Allogeneic T cells proliferated after co-culture with BAL DCs from sarcoidosis patients similarly to that of controls.[221] Using mo-DCs from blood and magnetic bead isolated blood DCs, on the other hand, induced less T cell proliferation, suggesting anergy of DCs from sarcoidosis patients.[222, 223] The heterogeneity of these

results may be due to the differences in the isolation method. Thus, standardized protocols for cell isolation from blood and BAL are required in order to ensure homogenous cell populations.[224, 225]

The use of well-defined DC subsets and their capacity to induce T cell proliferation could give an answer to the heterogeneity of T cells present in LS and non-LS sarcoidosis patients. As an example, IRF4$^+$ cDC2s were shown to activate Th17 cells in the pulmonary and intestinal mucosa in inflammatory and infectious diseases.[55, 116, 226] Since a Th17 phenotype was observed in sarcoidosis, it is of interest to address if cDC2s or any other MNP subset contribute to the skewing of Th17 cells. A summary of MNPs contributing to pathogenesis in sarcoidosis is illustrated in **Figure 7**.

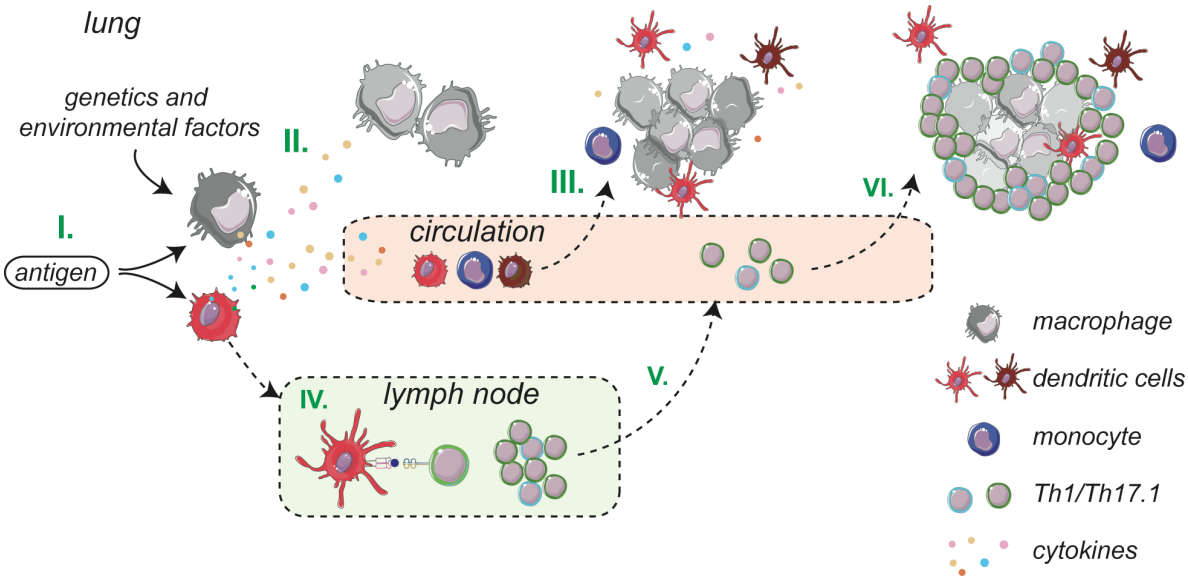

Figure 7. Sarcoidosis pathogenesis. A yet unknown antigen is taken up by MNPs of the respiratory tract **(I)**. The reaction of MNPs to the antigen is influenced by genetic and environmental factors. Upon uptake, MNPs produce cytokines that are released locally and into the blood stream **(II)**. This results in local and systemic inflammation and recruitment of MNPs to the site of inflammation **(III)**. Exposed macrophages are not able to clear the antigen and accumulate due to continuous activation. At the same time, DCs migrate with the antigen to the lymph node to activate naïve T cells **(IV).** Proliferating T cells differentiate into Th1 and Th17.1 cells and are released into circulation **(V**). In the lungs, T cells migrate to the site of inflammation. Here, T cells surround the non-necrotic core of macrophages **(VI)**.

Taken together, MNPs play an important role in sarcoidosis by producing cytokines, forming granulomas and activating T cells. As MNP subsets are highly specialized, detailed phenotypic and functional studies are required to dissect the MNP network in the sarcoidosis-affected tissue, and in blood.

5.2.5 Mononuclear phagocytes in tuberculosis

Sarcoidosis presents with non-necrotizing granulomas in contrast to tuberculosis (TB), that has necrotic granulomas caused by *Mycobacterium tuberculosis (Mtb*). The pathogenesis of TB is better understood than sarcoidosis due to high prevalence of TB and the opportunity to use animal models. The knowledge about MNPs derived from TB research may help to understand their function and the mechanisms involved, which could also facilitate insight into the complex pathogenesis of sarcoidosis.

TB is the leading cause of death from a single infectious agent. Every year, 10 million people get infected. About 1.5 million people died of the consequences of the disease in 2018 but the mortality seems to have a descending trend.[227] Despite currently available antibiotic treatments, drug resistances are increasing highlighting the necessity for new and additional treatment options and/or vaccination strategies.

A special role is attributed to MNPs in *Mtb* infection as macrophages serve as a reservoir and form necrotizing granulomas in the lung parenchyma.[228] Upon infection with *Mtb*, AMs as well as pulmonary DCs take up the bacteria and release proinflammatory cytokines such as TNF with its dual role contributing to disease severity as discussed above.[228-231] Additionally, a variety of chemokines are released to recruit monocytes, DCs and neutrophils to the site of infection, that potently phagocytose the bacteria.[228] However, *Mtb* has developed strategies to escape the immune system by inhibiting the fusion of phagosome and lysosome, inducing an anti-inflammatory program in macrophages and inhibiting the production of reactive oxygen species.[232, 233] Protected within the phagosome, *Mtb* replicates and ultimately leads to the necrosis of macrophages.[234] At the same time, DCs also get infected resulting in the maturation of DCs and their migration to the draining lymph node, where they activate naïve T cells.[235, 236] DCs mainly produce IL-12 and IL-23 skewing $CD4^+$ T cells mainly to a Th1 type.[237, 238] TB-specific Th1 cells are recruited to the site of infection where they accumulate around the necrotizing macrophages trying to clear the tissue from infection and produce large quantities of IFNγ.[232] The bacteria remain contained but alive within the necrotic core protecting the surrounding tissue. At this stage, patients suffer from a latent tuberculosis.

Using this extensive knowledge on MNPs in TB can hint to pathways involved in the pathogenesis that ultimately will improve our understanding of sarcoidosis pathogenesis.

6 MATERIAL AND METHODS

A detailed description of the materials and methods used in this thesis can be found in the respective Papers I-IV. The following section is a general description of the core methods and experimental setups used in this thesis.

6.1 ETHICAL CONSIDERATIONS

In the studies presented in this thesis, all samples were obtained from human blood and tissues. All studies have been reviewed and approved by the regional ethical review boards in Stockholm and Umeå and were conducted according to the Declaration of Helsinki. Prior to participation in our studies, study subjects gave oral and written informed consent. While sarcoidosis patients underwent the clinical procedure for diagnostic purposes, healthy subjects were volunteering for bronchoscopy. Volunteers were treated similarly to patients and, in addition, were compensated for their participation. For all study subjects, we handled sensitive data from individual persons. Thus, a coding system was used to blind personal data from the patient sample at any time.

6.2 STUDY SUBJECTS AND SAMPLE COLLECTION

For **all Papers**, healthy volunteers and patients with suspected sarcoidosis were recruited to either Norrlands University Hospital, Umeå or Karolinska University Hospital, Stockholm to undergo bronchoscopy. The bronchoscopies were performed by well-trained and experienced teams of nurses and physicians. A flexible bronchoscope was inserted through either nose or mouth into the airways. During the bronchoscopy, endobronchial biopsies (EBB) were taken from the major carinas of the bronchial tree. Next, a bronchial wash (BW) was performed on the contralateral side followed by a bronchoalveolar lavage (BAL) with saline solution to sample the proximal and distal bronchial tree, respectively. Additionally, in sarcoidosis patients, aspirates from the lung-draining lymph nodes (LLN) were taken using endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA). From all subjects, blood samples were taken prior to bronchoscopy (**Figure 8**).

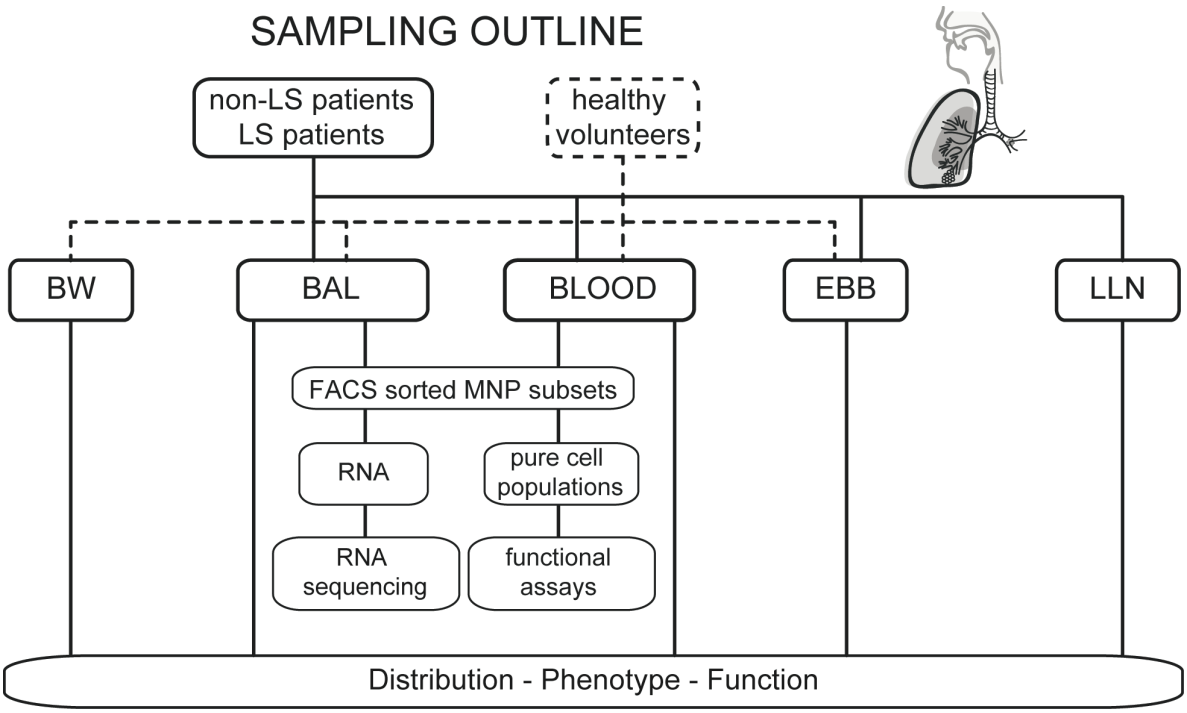

Figure 8. Sampling of sarcoidosis patients and healthy controls. In the studies, healthy controls as well as LS and non-LS sarcoidosis patients were sampled. Common for all studies, blood was sampled and peripheral blood mononuclear cells (PBMCs) isolated. During bronchoscopy, bronchial wash (BW), bronchoalveolar lavage (BAL) and endobronchial biopsies (EBB) were sampled. In sarcoidosis patients, the lung-draining lymph nodes (LLN) were sampled using endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA).

6.3 PROCESSING OF BLOOD, BW, BAL, EBB AND LLN

In order to obtain single-cell suspensions for flow cytometric analysis and cell culture, human tissue samples were processed. For **Paper I** and **II**, blood was collected in CPT tubes (BD) and peripheral blood mononuclear cells (PBMC) isolated according to the manufacturer's instructions. Blood **in Paper III** and **IV** was collected in heparin tubes and plasma collected by centrifugation for 10 min at 600 x *g*. Whole blood was diluted 1:1 with PBS and layered over Ficoll followed by density centrifugation at 900 x *g* for 25 min without brake.

EBBs had a size of around 1mm^3 and up to five specimens from the same subject were pooled and incubated with 1,4-Dithiothreitol (DTT) followed by digestion with collagenase and DNase to obtain a single-cell suspension. For **Paper I-IV**, BAL and BW were filtered and washed with PBS. In **Paper II**, up to five LLN aspirates were pooled, washed and due to red blood cell (RBC) contamination treated with RBC lysis buffer. Single-cell suspensions from blood, BAL, BW, EBB and LLN were counted and viability assessed using trypan blue exclusion prior to downstream applications.

6.4 FLOW CYTOMETRY AND FLUORESCENCE-ACTIVATED CELL SORTING

Essential to **Papers I-IV**, was the use of flow cytometry-based analyzers (LSRII and LSRFortessa, BD) and sorters (AriaIII and AriaFusion, BD). Flow cytometry has revolutionized the field of immunology and has become obsolete in research and clinical diagnostics. Common to flow cytometry is the laser excitation of fluorochromes, typically conjugated to antibodies targeting surface or intracellular epitopes, that emit light at a higher wavelength that is detected by specialized photomultiplier tubes. An addition in cell sorters compared to the analyzers is that samples can be further used after the staining as cells are deflected by electrodes and collected in tubes.

For staining of surface antigens, single-cell suspensions were incubated with a fixable viability dye followed by validated panels of fluorochrome conjugated antibodies. For staining of intracellular epitopes, cells were first fixed and permeabilized before incubation with antibodies targeting cytokines and transcription factors.

6.5 *IN VITRO* STIMULATION OF MNPs

In **Paper I** and **III**, MNPs from blood and BAL were cultured for 3h with or without LPS stimulation. In the presence of Brefeldin A (BFA), cytokines were allowed to accumulate that could be detected with an intracellular staining. BFA is a protein that inhibits protein transport from the endoplasmic reticulum to the GOLGI complex resulting in the accumulation of proteins in the cytoplasm.

6.6 RNA ISOLATION AND RNA SEQUENCING

For **Paper III**, RNA was isolated from FACS-sorted MNPs from blood and BAL to analyze the transcriptome using RNAsequencing (RNAseq). RNA was isolated from sorted cells and samples used for RNAseq required an RNA integrity number (RIN) higher than 6. Isolated RNA was transcribed to cDNA, amplified and a library created. Sequencing was performed using a HiSeq-4000 platform (Illumina) with a 2x150-bp pair-end sequencing with a depth of 20 million reads/sample. Sequencing quality was assessed using FASTQC and further analysis performed using R.

6.7 T CELL CO-CULTURE

For **Paper IV**, FACS-sorted MNPs from blood and BAL were cultured with allogeneic T cells from a healthy donor. For this, blood from a buffy coat was used and T cell isolated using the RosetteSep T cell enrichment kit (StemCell Technologies). To detect proliferation, T cells were labeled with 0.5 μM carboxyfluorescein succinimidyl ester (CFSE, Life Technologies). MNPs were co-cultured with CFSE labeled T cells for 5 days in a ratio of 1:50. On day 5, phorbol 12-myristate 13-acetate (PMA, 50ug/ml) and ionomycin (1ug/ml) were added to the culture in the presence of BFA (10ug/ml) for 4h to assess intracellular cytokine production.

7 RESULTS AND DISCUSSION

This thesis was designed to characterize human mononuclear phagocytes (MNPs) in the respiratory tract during steady state and sarcoidosis. For this, we first determined the distribution of MNPs during steady state conditions (**Paper I**). Our detailed approach by sampling MNPs from the bronchial tissue, the luminal site of the bronchi (lavages), and blood identified differences in MNP distribution both within the respiratory compartment as well as in comparison to blood.

In order to answer if MNPs are involved in sarcoidosis pathogenesis, we first determined how MNPs are distributed in the same respiratory compartments in sarcoidosis patients with and without Löfgren's syndrome (LS and non-LS) (**Paper II**) and compared it to healthy controls (HCs) (**Paper III**). Here, we observed important differences between non-LS sarcoidosis patients and HCs. In non-LS patients, monocytes were the dominating cell subset compared to dendritic cells (DCs). Functionally, we observed a highly pro-inflammatory profile in non-LS patients compared to LS patients and HCs. In particular monocytes showed high expression of genes related to inflammation and of the proinflammatory cytokine tumor necrosis factor (TNF). Interestingly, TNF production by pulmonary monocytes predicted which patients at time of diagnosis are at risk to develop severe disease (**Paper III**). Additionally, we found respiratory MNPs to induce Th1 cells with potent interferon gamma production (**Paper IV**). Taken together, these results highlight the importance of studying respiratory MNPs in detail and identifies MNPs as main drivers of pathogenesis in sarcoidosis. Detailed information is described in the following sections and in the original **Papers I-IV**.

7.1 IDENTIFICATION AND HETEROGENEITY OF RESPIRATORY MNPs

In order to compare monocytes and DCs across studies and compartments using flow cytometry, we applied similar staining panels and gating strategies as exemplified in **Figure 9A**. MNPs were identified as HLA-DR$^+$ and lineage negative. Lineage markers excluded T cells, B cells, NK cells, and neutrophils. Amongst MNPs, CD123$^+$ expression was used to identify plasmacytoid DCs (pDCs). CD11c$^+$ MNPs and expression of CD14 and CD16 identified classical, intermediate, and non-classical monocytes. Conventional DCs were defined as CD14$^-$CD16$^-$ and expression of CD141 and CD1c identified cDC1s and cDC2s, respectively (**Figure 9A**). In bronchoalveolar lavage (BAL), alveolar macrophages (AMs) were identified as side scatter high and by their autofluorescence. Using the same staining panel and gating strategy in blood and the respiratory tract, we used the same nomenclature for monocytes in both compartments. However, in the respiratory tract, it cannot be excluded that CD14$^+$ and CD16$^+$ monocytes may in fact be monocyte-derived cells.[111, 114, 115] Monocytes are highly plastic and upon migration to the tissues differentiate into

monocyte-derived dendritic cells (mo-DCs) or macrophages.[67] In BAL from HCs, mo-DCs were identified as CD14 and CD1c expressing cells and comprised about 30% of CD14$^+$ monocytes (**Figure 9B**). In LS and non-LS sarcoidosis patients, about 20% of CD14$^+$ cells in BAL expressed CD1c (**Figure 9B**). This might be the result of a higher migratory potential of monocytes to the respiratory tract as a response to the inflammation in sarcoidosis. Differences in frequencies of mo-DCs in sarcoidosis patients and HCs could indicate distinct functional properties.

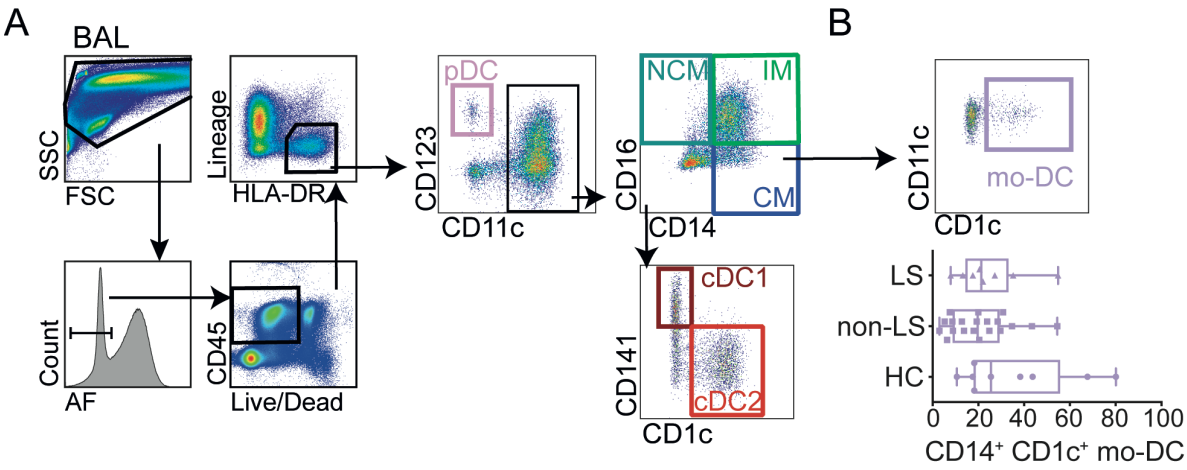

Figure 9. Phenotype of MNPs in bronchoalveolar lavage. (A) Representative plots of a non-LS patient to identify different MNP subsets in cells obtained by bronchoalveolar lavage (BAL). SSC: side scatter; FSC: forward scatter; AF: autofluorescence **(B)** Identification of mo-DCs as CD14$^+$ CD1c$^+$ cells in BAL. Graph shows the frequencies of mo-DCs in HCs compared to LS and non-LS patients.

In the different compartments of the respiratory tract as well as blood, monocytes and DCs could be identified at varying frequencies (**Figure 10A**). The most striking differences in distribution were observed comparing MNPs from blood and the respiratory tract. In HCs and sarcoidosis patients, frequencies of HLA-DR$^+$ MNPs increased from the proximal to the distal part of the lower airways. The alveoli are functionally important and responsible for the uptake of oxygen. To ensure organ function, MNPs could be preferentially seeded in the distal area to protect the epithelium from invading pathogens. The most common subset in blood were classical monocytes (CMs) that egress from the bone marrow and undergo differentiation into intermediate (IMs) and non-classical monocytes (NCMs).[70] In LS and non-LS sarcoidosis, frequencies of circulating IMs were elevated likely due to the persistent inflammation (**Figure 10B**). Differentiation of CMs to IMs is accelerated by inflammation and an increased frequency of blood IMs is observed in other infectious or inflammatory diseases including sarcoidosis.[215, 217, 239, 240] We also observed an increase of IMs in BAL of non-LS patients compared to HCs indicating local pulmonary inflammation in sarcoidosis (**Figure 10B**). Importantly, we identified IMs to be predictors of disease outcome. For this, we followed patients over two years after the diagnosis was established. We found that patients with high frequencies of blood IMs

at time of diagnosis were more likely to develop chronic disease than patients with lower frequencies of blood IMs at time of diagnosis (**Figure 10C**). This finding highlights the importance of dissecting the MNP network in the respiratory tract during sarcoidosis.

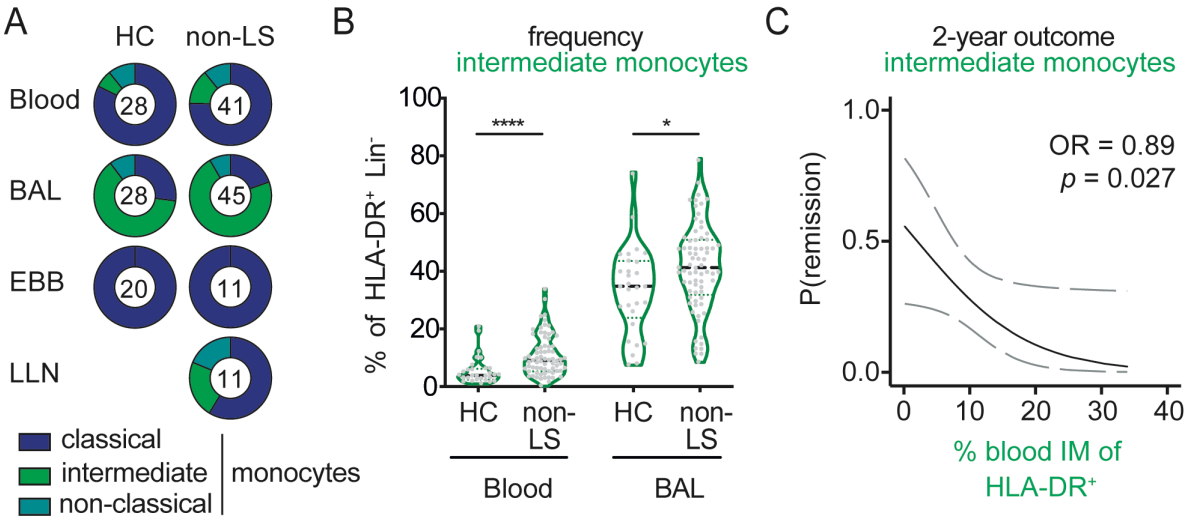

Figure 10. Distribution of monocytes in blood and the respiratory tract during steady state and sarcoidosis. (A) Pie charts show distribution of classical (blue), intermediate (green) and non-classical (teal) monocytes in blood, bronchoalveolar lavage (BAL), endobronchial biopsies (EBB), and lung-draining lymph nodes (LLN) in healthy controls (HCs) and non-LS sarcoidosis patients. Number of subjects included are indicated in the center of the pie chart. **(B)** Frequencies of intermediate monocytes in blood and BAL of HCs and non-LS sarcoidosis patients. Statistical analysis was performed using the non-parametric Mann Whitney U test. *$p < 0.05$, ****$p < 0.0001$. **(C)** Graph shows predictive modelling for frequencies of intermediate monocytes in blood of sarcoidosis patients at time of diagnosis using linear regression. OR: odds ratio.

Investigating the lung-draining lymph nodes (LLNs) in sarcoidosis is a unique possibility to study the site of T cell priming. We performed the first detailed analysis of MNPs in the LLN of LS and non-LS patients using flow cytometry.[214] As expected, MNPs in the LLN are rare (ca. 1% of total cells). Interestingly, we observed a difference in distribution of MNPs between LS and non-LS sarcoidosis patients, primarily caused by plasmacytoid DCs (pDCs). In non-LS patients, pDCs comprise of about 20% of HLA-DR$^+$ cells, while in LS patients it is only 5% on average. Activated pDCs produce type I interferons that in turn activate IFNγ producing Th1 cells, the main drivers of sarcoidosis.[196] This shift towards monocytes in the LLNs in LS patients compared to non-LS patients indicates important differences that may have an impact on disease outcome.[200]

In order to optimally interact with and activate T cells, MNPs have to upregulate surface expression of HLA-DR for antigen presentation and co-stimulatory receptors such as CD80 or CD86, a process called maturation.[6, 241] Overall, MNPs were most mature in the respiratory tract compared to blood. Surprisingly, expression of CD80

and HLA-DR was lower in LLNs compared to BAL in sarcoidosis patients. Increased maturation in the lung environment could indicate an ongoing inflammation leading to the maturation of MNPs.[242, 243] Supportive of this theory is the upregulation of CCR7, the chemokine receptor mediating the migration to the draining lymph nodes.[122]

In summary, there are distinct differences in distribution and phenotype between DCs and monocytes from blood, lung or lung-draining lymph nodes highlighting the importance of studying MNPs subsets side-by-side.

7.2 TRANSCRIPTOME OF PULMONARY MNPs DURING STEADY STATE AND SARCOIDOSIS

Flow cytometry is a powerful tool to reliably study immune cells. However, it also has its limitations. A caveat of flow cytometry is the use of predefined markers to identify MNPs. Particularly in tissues, monocytes and DCs share surface markers that in blood served to distinguish MNPs.[18] Using RNAsequencing (RNAseq) allows for an unbiased approach to study MNPs and with a higher resolution as the entire transcriptome is quantified. RNAseq analyses have revolutionized our understanding of cell heterogeneity, ontogeny and tissue specificity has revolutionized.[244] We performed RNAseq on whole populations of FACS-sorted monocytes and DCs from blood and BAL as well as AMs. The advantage of doing RNAseq on sorted MNP subsets compared to bulk sequencing of total BAL cells, is to find important signatures in rare subsets that would be masked by the abundance of AMs and T cells.[245-247]

Similar to the phenotype of MNPs, we observed pronounced differences in gene expression between blood and BAL MNPs independent of whether cells were derived from sarcoidosis patients or HCs. A total of 2603 genes were differentially regulated between blood and BAL MNPs **(Figure 11A)**. Common to all MNPs obtained from BAL, expression of genes related to the inflammatory response (*CD40*, *TNF*, *IL1A*, *CSFR1*, *NFKB1, CD274*), chemotaxis (*CCL2*, *CCL8*, *CCL19*, *CCL20, CCR6*), and cell differentiation (*CD40*, *CCR7*, *AXL*, *TGFBR1, CD207*) was higher compared to blood MNPs. However, also genes related to angiogenesis (*VEGFA*, *PDGFA*, *ITGAV*), coagulation (*CD9*, *C3*, *FN1*) and signaling pathways were upregulated as assessed by gene set enrichment analysis **(Figure 11B)**. We confirmed these RNAseq results on the protein level for CCR6, CCR7, and CD207 (langerin), all markers predominantly expressed by cDC2s. CCR6 is highly expressed by BAL cDC2s in both sarcoidosis patients and HCs. Its cognate ligand is CCL20, which is highly expressed by BAL MNPs suggesting that the recruitment of cDC2s is mediated by other pulmonary MNPs. Since $CCR6^{+}$ cDC2s are not restricted to sarcoidosis patients or HCs, it can be assumed that CCL20 is constitutively expressed by epithelial cells and MNPs.[248-251] The continuous recruitment of cDC2s could be a mechanism to rapidly respond to infiltrating pathogens to the lung tissue.[252]

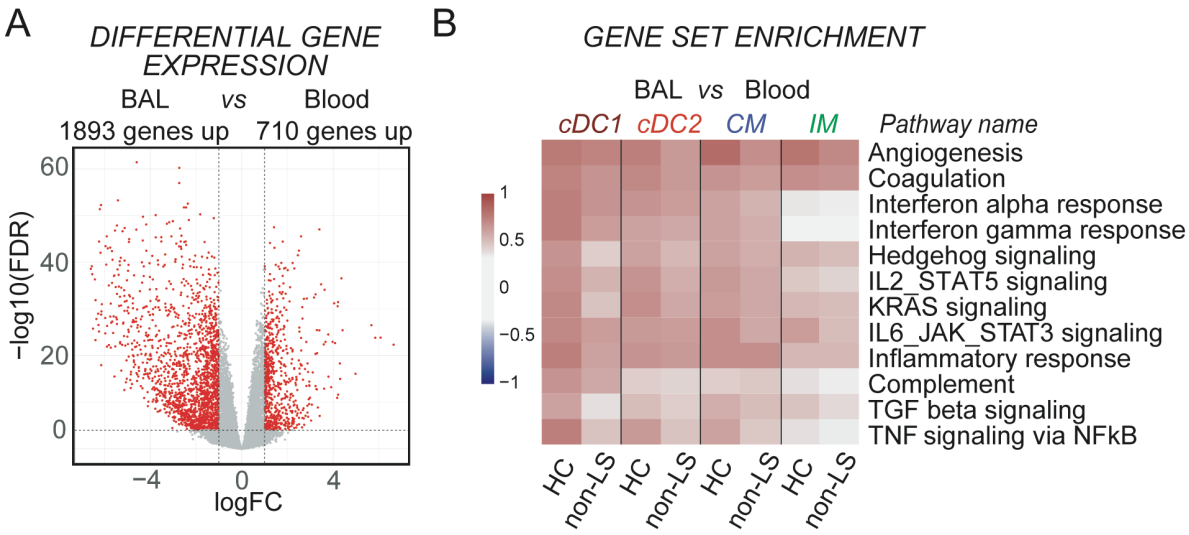

Figure 11. Differential gene expression of blood and BAL MNPs from HCs and non-LS patients. (A) Volcano plot shows differentially expressed genes (red dots) that are common to all MNP subsets from blood and BAL of healthy individuals and sarcoidosis patients. **(B)** Differentially expressed genes from different MNP subsets were allocated to pathways in the gene set enrichment analysis. MNPs between blood and BAL were compared in HCs and non-LS patients.

Next, we analyzed differences in gene expression in MNPs from sarcoidosis patients and HCs. Both in blood and BAL MNPs, we observed that genes related to the TNF and IL-6 pathways were upregulated in cells from sarcoidosis patients compared to HCs. Additionally, genes in response to IFNγ were upregulated in MNPs from sarcoidosis patients confirming previous observations.[253] This is likely a response to the excessive activation of T cells in sarcoidosis and highlights the key role of T cells in sarcoidosis. Particularly, genes of the TNF pathway were highly upregulated in monocytes of non-LS patients compared to HCs. In contrast, AMs showed upregulated genes related to the reactive oxygen species pathway, TGF-β or the fatty acid metabolism. Interestingly, *CSF1* was upregulated by MNPs in BAL of non-LS patients that favor the differentiation of alternatively activated macrophages.[254] Supportive of this, MNPs in BAL of non-LS patients also expressed high levels of TGF-β compared to controls, also favoring alternatively activated macrophage polarization.[255] Here, future analyses using single-cell RNAseq may reveal the heterogeneity of AMs in sarcoidosis as shown for other pulmonary diseases.[256-261] It is likely that the balance between pro- and anti-inflammatory macrophages contribute to disease progression and resolution. Collectively, these findings mark significant differences between AMs and monocytes in the lungs of sarcoidosis patients.

7.3 FUNCTIONAL PROPERTIES OF MNPs DURING STEADY STATE AND SARCOIDOSIS

To confirm this inflammatory profile, we performed functional experiments of MNPs and assessed TNF expression on a protein level in monocytes, macrophages and DCs. Cells from blood and BAL were cultured for 3 hours with lipopolysaccharide (LPS) or left unstimulated in the presence of BFA to allow accumulation of cytokines intracellularly. As expected, blood monocytes from HCs and sarcoidosis patients produced TNF only after LPS stimulation. In contrast, BAL monocytes produced TNF also without stimulation. Intrinsic TNF production in sarcoidosis patients was observed before and was mostly attributed to macrophages.[207, 208, 262] The advantage of our experimental setup is the use of flow cytometry compared to ELISA used in previous studies. It was assumed that AMs are the major source of TNF as those are the most abundant cell type in BAL.[211] However, our detailed approach to determine TNF production by individual MNP subsets, in fact, showed that higher frequencies of pulmonary monocytes rather than AMs or DCs produced TNF (**Figure 12A**). Additionally, frequencies of unstimulated TNF producing BAL monocytes was significantly higher in non-LS patients compared to HCs and LS patients. Importantly, non-LS patients with high frequencies of TNF producing intermediate monocytes were more likely to develop progressive disease in a prediction model (**Figure 12B**). We did not find that AMs predicted disease outcome as previously described.[209] These data highlight the importance of studying pulmonary monocytes in sarcoidosis. Even though monocytes and DCs are rare cells in the lungs compared to AMs, they contribute significantly to disease pathogenesis in sarcoidosis.

In contrast, we found LS sarcoidosis patients to have more IL-6 producing monocytes compared to non-LS patients. Comparing intrinsic TNF and IL-6 production by monocytes showed a positive correlation in non-LS patients while there was a trend towards a negative correlation in LS patients (**Figure 12C**). These data suggest a distinct cytokine profile in LS and non-LS sarcoidosis patients indicating differences in the pathogenesis. This has raised the question to view LS and non-LS sarcoidosis as two distinct disease entities.[12] IL-6 is a driver of Th17 cell differentiation, which were shown to be accumulated in LS patients.[197, 263]

Anti-TNF treatment is currently used as a 3rd line treatment in sarcoidosis. Previous studies showed that patients with high serum TNF concentrations and higher numbers of circulating intermediate monocytes are more likely to benefit from anti-TNF treatment.[210, 264, 265] In our studies, we aimed to determine if intrinsic TNF expression can also be used to predict success of anti-TNF therapy. Despite few patient numbers at this time, our preliminary data is interesting. We measured TNF expression by pulmonary MNPs in patients with progressive disease prior to and after anti-TNF treatment (infliximab). Prior to anti-TNF treatment, we found a range of intrinsically TNF producing MNPs in the patients that may be skewed due to their treatment with

corticosteroids at that time. To our surprise, after anti-TNF treatment, we observed that monocytes from BAL of all patients expressed TNF without stimulation (on average 60% of monocytes) (**Figure 12D**). These data indicate that despite the anti-inflammatory treatment, cells are still highly inflammatory and may be one reason for a relapse after discontinuation of anti-TNF treatment.[266] TNF expression by pulmonary monocytes may therefore be a tool to determine duration of anti-TNF treatment. Anti-TNF treatment is well established in rheumatoid arthritis and Crohn's disease.[267, 268] Among the risks of treatment with biologicals is, however, the development of anti-drug antibodies (ADA).[269] To prevent ADA production, low-dose corticosteroids or methotrexate accompany anti-TNF treatment resulting in further side effects.[270] Thus, it is crucial to identify sarcoidosis patients that would suffer from severe disease and initiate targeted treatment early on to prevent excessive inflammation.

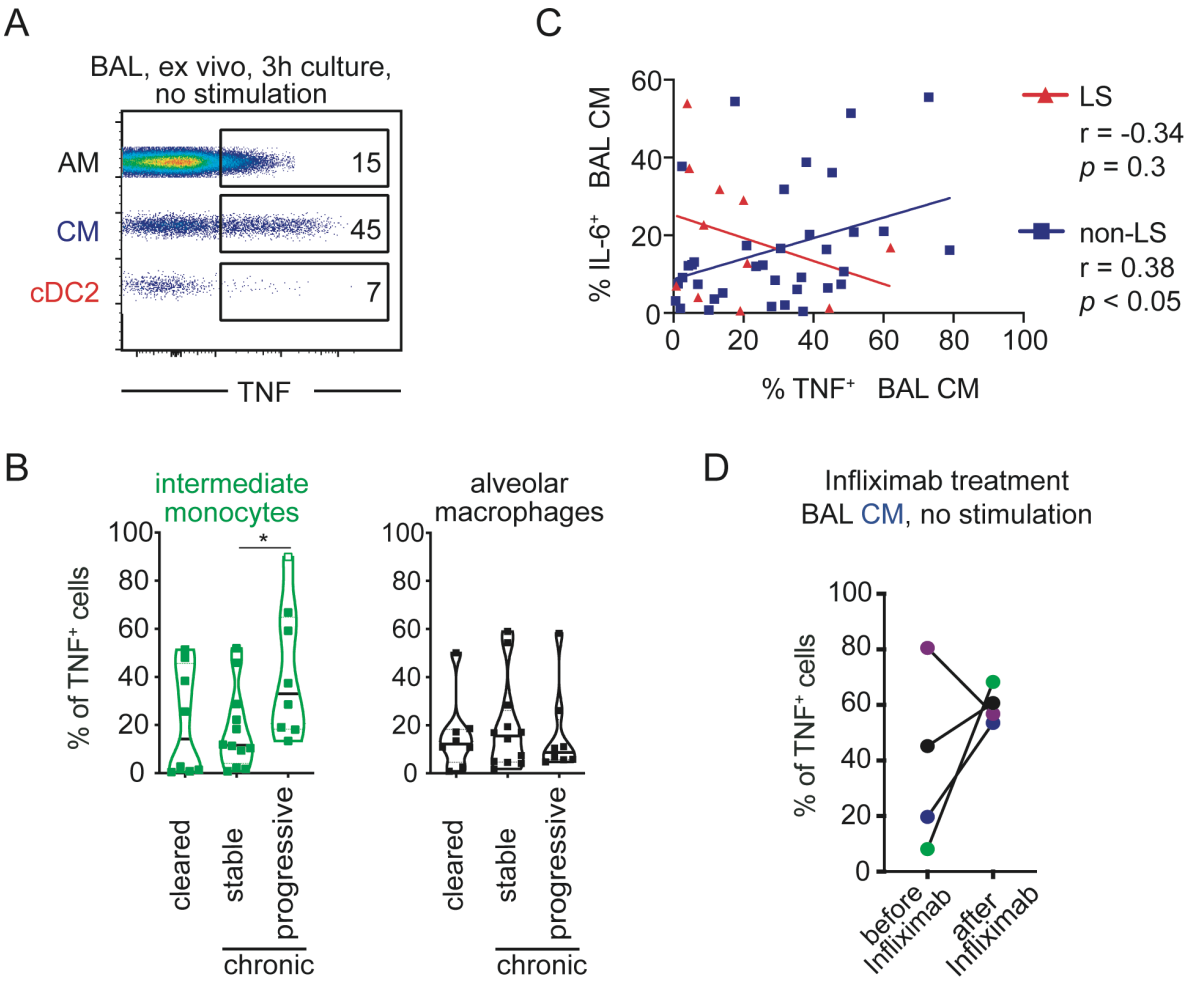

Figure 12. TNF expression by respiratory MNPs in non-LS sarcoidosis patients. (A) Concatenated pseudocolor plot shows intracellular TNF expression in AMs, CMs and cDC2s from one non-LS patient. Cells were cultured for 3h without stimulation. **(B)** Violin plots show TNF expression by unstimulated IMs and AMs at time of diagnosis and grouped based on the clinical outcome after two years. **(C)** Correlation of IL-6 and TNF expression by BAL CMs in non-LS (blue squares) and LS patients (red triangles). **(D)** Intracellular TNF expression by BAL CMs in non-LS patients prior to infliximab treatment and after six months. Patients are color-coded and the line connects each patient before and after infliximab treatment.

T cells are expanded in sarcoidosis patients and are likely critical in pathogenesis as they are a major source of IFNγ, that is abundantly expressed in sarcoidosis patients. MNPs as antigen-presenting cells are responsible for T cell activation. Hence, we wanted to assess the ability of MNPs from sarcoidosis patients to induce T cell proliferation. FACS-sorted MNPs from blood and BAL of non-LS patients were co-cultured with allogeneic T cells from a healthy donor. Overall, MNPs from blood induced more T cell proliferation than the respective MNP subset from BAL (**Figure 13A+B**). Blood and BAL cDC2s were altogether better at inducing T cell proliferation compared to cDC1s followed by classical and intermediate monocytes and alveolar macrophages that did not induce T cell proliferation (**Figure 13B**). An exception were classical monocytes from BAL, that potently induced T cell proliferation. This could be due to the presence of $CD1c^+$ mo-DCs among the $CD14^+$ monocytes. Thus, it cannot be excluded that a fraction of cells comprised of mo-DCs that are superior in inducing T cell proliferation compared to tissue monocytes.[115] In our cohort, 20% of monocytes were mo-DCs as assessed by CD1c expression **(Figure 9B)**. Despite the low frequency of mo-DCs, their ability to induce T cell proliferation was similar to *bona fide* DCs. This highlights another important feature of monocytes and monocyte-derived cells in sarcoidosis.

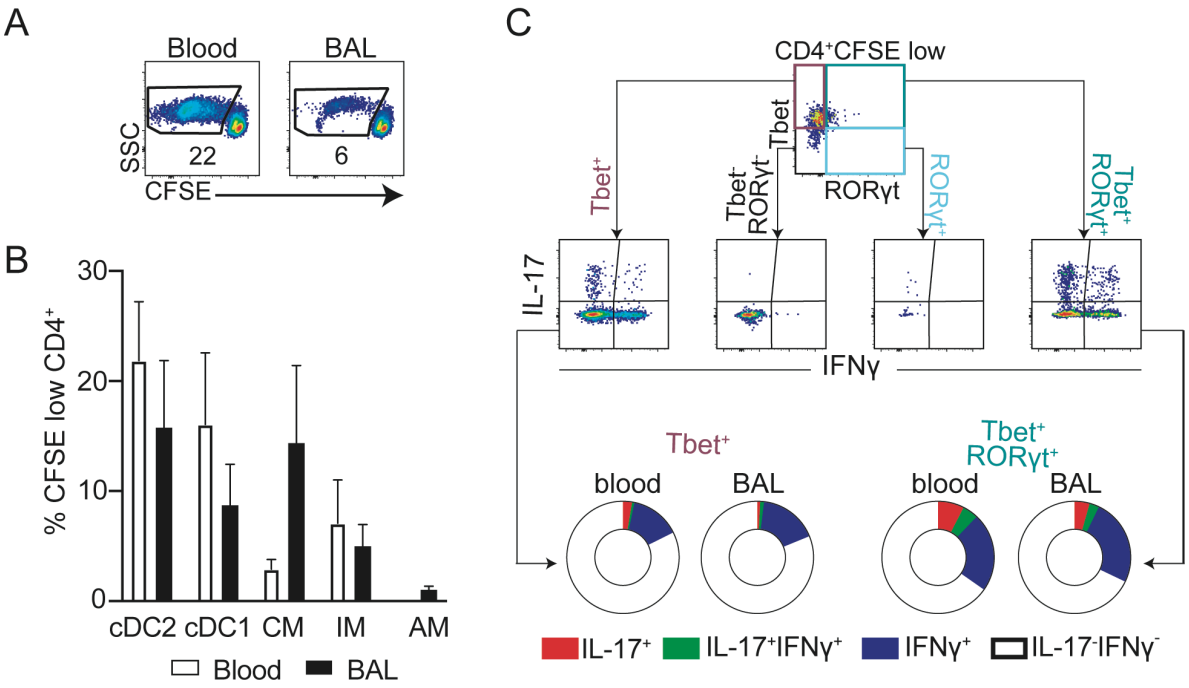

Figure 13. T cell proliferation and differentiation induced by BAL MNPs. (A) Representative plot of T cell proliferation assessed by CFSE dilution after co-culture of blood and BAL cDC2s with allogeneic T cells. **(B)** Summary of induced T cell proliferation after co-culture with MNP subsets from blood and BAL. Bar graphs show mean ± SEM. **(C)** Representative plot shows expression of transcription factors Tbet and RORγt in proliferated $CD4^+$ T cells after co-culture with BAL cDC2s. Further, plots show IL-17 and IFNγ expression after restimulation with PMA/ionomycin. Pie charts illustrate the cytokine expression in proliferated $Tbet^+$ and $Tbet^+RORγt^+$ cells.

Further, we wanted to assess how T cells differentiate upon activation by MNPs from sarcoidosis patients. We hypothesized that MNPs should induce T cells similar to those found in BAL of sarcoidosis patients. These T cells characterized by their expression of CXCR3 and CCR6 are termed Th17.1 cells and excelled at producing IFNγ.[198, 199, 271, 272] As shown in the respective studies, chemokine receptors are co-expressed by Th1 and Th17 cells.[198, 199] Thus, we used Th1 and Th17 specific transcription factors and cytokines instead to determine the T cell fate.[273, 274] Blood and BAL MNPs from sarcoidosis patients mostly induced Tbet expressing Th1 cells producing mainly IFNγ (**Figure 13C**). Between 5-10% of proliferated T cells also expressed Tbet together with RORγt. Upon restimulation, T cells mostly produced IFNγ followed by IL-17 suggesting the presence of Th17.1 cells as observed *in vivo* (**Figure 13C**).[198, 199] If those cells resemble Th1 cells that acquire a Th17-like phenotype or *vice versa* remains to be elucidated in further studies. However, this data highlights the plasticity of T cells, particularly during inflammatory conditions.[275, 276] Hence, it is crucial to also understand how MNPs are involved in determining the T cell fate. Altogether, we could show the potential of MNPs from sarcoidosis patients to induce pathogenic Th17.1 cells *in vitro.* This offers a novel way to study this subset of T cells in the pathogenesis of sarcoidosis.

8 CONCLUSIONS

The studies presented in this thesis were designed to unravel the distribution and function of human mononuclear phagocytes (MNPs) in the respiratory tract during steady state and sarcoidosis. Pulmonary MNPs serve as gatekeepers in the lungs as they constantly face the challenge to either maintain tolerance or initiate immune responses. The role of MNPs in tissues has been extensively studied in mouse models. However, detailed knowledge about human pulmonary MNPs is scarce as tissues are often acquired from patients with pulmonary diseases. Hence, retrieving samples from healthy volunteers from different anatomical locations along the respiratory tract will pave the way to unravel the complexity of MNPs in the lungs. Understanding the distribution and function of MNP subsets during steady state is necessary to identify factors involved in pulmonary diseases, such as sarcoidosis. In sarcoidosis, reliable mouse models are still lacking that could identify pathways involved in disease pathogenesis. Hence, an in-depth characterization of MNPs from the respiratory tract comparing steady state and sarcoidosis will help uncover mechanisms involved in disease pathogenesis. This will help to develop new therapy options and improve the patient's quality of life.

Altogether, the main conclusions of this thesis are as follows:

- MNPs have a distinct distribution, phenotype and transcriptome based on their anatomical location with major differences between blood and the respiratory tract during both steady state and sarcoidosis (**Paper I** + **II** + **III**).

- The distribution of monocytes and DCs in sarcoidosis helps to predict which patients will develop progressive disease (**Paper III**).

- Pulmonary monocytes contribute significantly to inflammation in sarcoidosis by producing TNF, which is also associated with disease progression (**Paper III**).

- Circulating and pulmonary DCs from sarcoidosis patients effectively induce T cell proliferation with a Th1 phenotype (**Paper IV**).

9 FUTURE DIRECTIONS

The characterization of pulmonary MNPs during steady state and sarcoidosis as outlined in this thesis is a first step towards improving our knowledge on how the lungs maintain homeostasis and react during immune challenges. However, several questions remain that should be addressed in future studies.

We have successfully established methods to characterize MNPs in the human respiratory tract. The identification of MNPs was based on surface receptors found in blood that could be detected by flow cytometry. We and others have detected overlapping phenotypic characteristics between MNPs in the respiratory tract.[114, 115, 129, 277] Hence, a more unbiased approach using single-cell RNAseq would help to characterize MNP populations in-depth as described for circulating MNPs.[94-96, 244] Together with bioinformatic tools, the mononuclear phagocyte landscape will change and likely reveal even more heterogeneity.[278, 279]

A detailed study on a single-cell level comparing MNPs from the alveolar space with MNPs from the lung parenchyma would be of interest. This could address the question to which extent cells retrieved by bronchoalveolar lavage reflect cells from the lung parenchyma. When studying immune cells on a single-cell level in the lungs, it would also be of interest to study the kinetics and migration of MNPs to and from the tissue. Upon controlled experimental inflammation[280, 281] or infection,[282] studies using labeled cells[70, 283] would allow to track single-cells to determine their potential to migrate into tissues. This could also answer the question if MNPs transmigrate through the epithelial layer to the draining lymph nodes.

As a follow-up to the transcriptome analysis, pathways related to the energy metabolism, coagulation, and signaling pathways related to cell proliferation of MNPs from sarcoidosis patients can be further explored. That could answer at what stage the fate of sarcoidosis patients are skewed towards either disease resolution or disease progression.

One major remaining challenge in sarcoidosis research remains to identify the antigen(s) that initiate(s) disease and result in granuloma formation. Identification of one or several antigens could help explain the differences observed in cytokine production by MNPs in LS and non-LS sarcoidosis patients. MNPs could be specifically stimulated with the antigen that would result in a distinct T cell response. Currently in use are mouse models and *in vitro* systems based on candidate antigens.[284] Studying the immunopathogenesis of sarcoidosis in model systems allows for the identification of novel factors contributing to disease progression. That knowledge could be exploited to develop therapeutic interventions to help the immune system clear the antigen and ultimately resolve inflammation.

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